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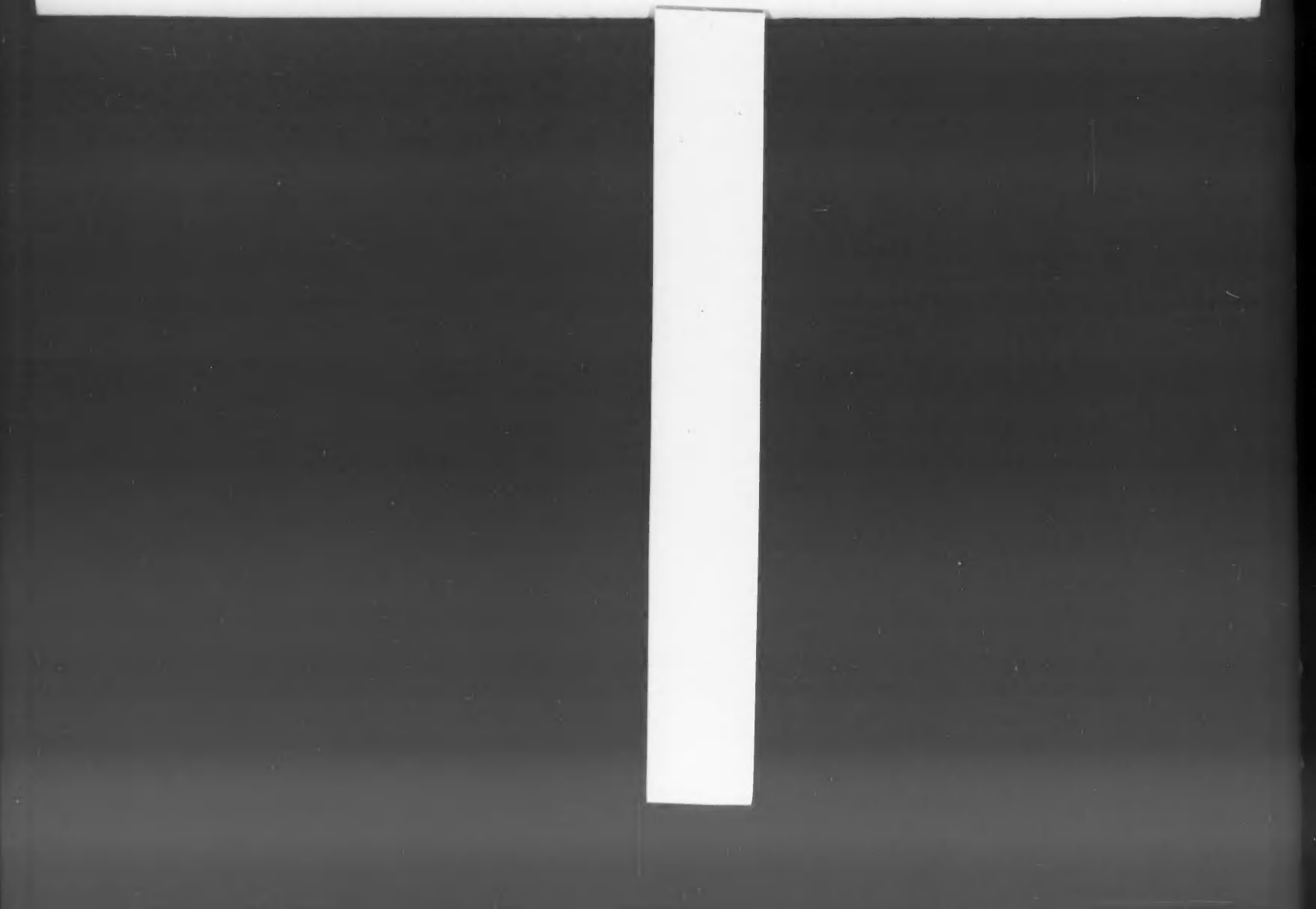

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Metabolism of Hexose Phosphate Esters

1. METABOLISM IN NORMAL AND ALLOXAN-DIABETIC RABBITS

2. METABOLISM IN ISOLATED RAT DIAPHRAGM AND THE INFLUENCE OF INSULIN

By ANNE BELOFF-CHAIN, E. B. CHAIN, D. BOVET, F. POCCHIARI, R. CATANZARO and L. LONGINOTTI
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(*Received* 30 *June* 1952)

Shortly after the discovery of insulin, it was shown by a number of investigators (Wigglesworth, Woodrow, Smith & Winter, 1923; Harrop & Benedict, 1924; Bollinger & Hartman, 1925) that there was a decrease in the inorganic phosphorus content of the blood in normal and diabetic animals following the injection of insulin. These observations first led to the hypothesis that insulin was concerned with the phosphorylation of carbohydrates, and a number of investigators tried to correlate the decrease in the inorganic phosphorus of the blood with an increase in the hexose monophosphate of muscle. Cori & Cori (1932) and Soskin, Levine & Hechter (1941), however, considered that the increase in hexose monophosphate of muscle is probably a secondary effect of insulin due to an output of adrenaline. Nelson, Rapoport, Guest & Mirsky (1942) suggested that the decrease in inorganic phosphorus of the blood following insulin injections could be accounted for at least in part by the increase in the acid-soluble phosphorus fraction of the liver.

Kaplan & Greenberg (1943, 1944), working with radioactive phosphate, concluded from their results that insulin accelerates the transfer of phosphorus from the blood stream to the tissues, increases the hexose monophosphate content of muscle, and (in agreement with the results obtained by Nelson *et al.* 1942) increases the phosphorylation processes in the liver, as shown by the increased incorporation of the injected radioactive phosphorus into the acid-soluble phosphorus fraction and into the labile phosphate groups of adenosine triphosphate.

Sacks (1945), also using radioactive phosphorus, studied the effect of insulin on phosphate turnover in cat striated muscle. He reported an increased turnover rate of phosphocreatine, adenosine triphosphate and glucose 6-phosphate. Haugaard, Marsh & Stadie (1951) studied the changes in the phosphorylated intermediates in rat diaphragm muscle after incubation in a glucose-containing medium in the presence and absence of insulin. They concluded from their results that insulin greatly increases the turnover rate of high-energy phosphate.

Price, Cori & Colowick (1945) reported that the hexokinase reaction in muscle and brain extracts was inhibited in rats by previously injecting them with anterior pituitary extracts or by adding anterior pituitary extracts to *in vitro* preparations, and that this inhibition was released by insulin. They also reported that the hexokinase from alloxan-diabetic rats is of the same order as that obtained in preparations from normal rats previously injected with anterior pituitary extracts, and that the activity is again restored to normal by insulin. These observations produced a great deal of speculation that the action of insulin is concerned with the phosphorylation of glucose to glucose 6-phosphate, i.e. the hexokinase reaction. However, a number of investigators (see Stadie, Haugaard & Hills, 1950, for references) were unable to confirm the work of Price *et al.* (1945), and up to the present there is no conclusive evidence for the action of insulin being directly involved in the phosphorylation of glucose.

The work reported in the present paper was undertaken to obtain some further evidence for or against this much disputed hypothesis for the site of action of insulin in carbohydrate metabolism. Two lines of approach were adopted: (1) the *in vivo* metabolism of glucose 1-phosphate and glucose 6-phosphate was compared with that of glucose in normal and alloxan-diabetic rabbits* and (2) the *in vitro* metabolism by the isolated rat diaphragm muscle of some of the phosphorylated hexoses was compared with that of glucose in the presence and absence of insulin.

* Some of these results have been previously published in a preliminary note (Beloff-Chain, Bovet, Chain & Pocchiari, 1951). After our preliminary publication Kuether (1951) reported results of similar but less detailed experiments carried out with glucose 1-phosphate on alloxan-diabetic rats. His findings and conclusions were very similar to our own.

Studies with the isolated diaphragm from alloxan-diabetic rats are now in progress and will be reported later.

METHODS AND MATERIAL

Preparations

Glucose 1-*phosphate* (*G*1-*P*). The dipotassium salt was prepared according to the method of McCready & Hassid (1944) using the cation-exchange resin Zeo-Karb 215 and the anion-exchange resin De-Acidite B, or the corresponding Amberlite resins.

Glucose 6-*phosphate* (*G*6-*P*). The barium salt was prepared according to the method of Seegmiller & Horecker (1951) from glucose and tetraphosphoric acid (Cherbuliez & Weniger, 1946).

Fructose 1:6-*diphosphate* (*F*1:6-*P*). The barium salt was prepared according to the method of Neuberg & Lustig (1942) from brewer's yeast.

Fructose 6-*phosphate* (*F*6-*P*). The barium salt was prepared from F1:6-P by hydrolysis in N-HBr for 5 days at 32° according to the method of Neuberg, Lustig & Rothenberg (1943).

Preparation of sodium salts. The hexose phosphates were used in all experiments as solutions of the sodium salts.

The sodium salt of G1-P was prepared by passing a solution of the K salt through a cation-exchange resin, and washing the resin with water until the pH of the effluent was approximately 4. The solution was then adjusted to pH 7 with a few drops of 30% NaOH, and concentrated *in vacuo* to the desired volume.

The sodium salts of G6-P, F1:6-P and F6-P were prepared by adding a slight excess of Na_2SO_4 to a solution of the barium salt (in dilute acid in the case of F1:6-P); the barium sulphate was centrifuged off and washed twice with water, and the supernatant plus the washings were adjusted to pH 7 and concentrated *in vacuo* to the required volume.

Analytical methods

Glucose was determined by the method of Miller & Van Slyke (1936).

All P determinations were made using the method of Berenblum & Chain (1938).

Glycogen was determined using the procedure of Good, Kramer & Somogyi (1933) as modified by Stadie, Haugaard & Marsh (1951).

G1-P was determined in the protein-free filtrates of blood and in the urine by measuring the inorganic P present before and after 10 min. hydrolysis in N-H_2SO_4 at 100°. In the *in vitro* experiments the G1-P was determined by measuring the glucose liberated after a similar hydrolysis.

G6-P was determined by subtracting the inorganic P from the total P obtained on ashing in perchloric acid.

In the *in vivo* experiments corrections were made for the initial values obtained in the blood prior to the injection of the substances.

Although the methods used for the determination of G1-P and G6-P are not strictly specific it was considered that under the conditions of these experiments they substantially measure the phosphate esters under investigation.

F1:6-P and F6-P were determined by measuring the fructose according to the method of Roe (1934).

The concentrations of the hexose phosphates are expressed throughout as mg. of glucose or fructose present.

Procedure for in vivo *experiments*

Male rabbits, weighing approximately 2 kg., were used. To produce alloxan diabetes, a single injection (200 mg./kg. in 5% solution) of alloxan was given intravenously into the ear vein. During the hypoglycaemic period following this injection the animals were fed a high carbohydrate diet and given concentrated glucose solutions to drink. Blood glucose was determined on the third or fourth day after treatment and subsequently for a minimum period of 4 days prior to an experiment. Only animals with a blood-glucose level exceeding 250 mg./100 ml. biood for a minimum period of 4 days were considered as diabetic. The animals were kept on a diet consisting only of green vegetables for 24 hr. before an experiment, or for a period of 48 hr. in some experiments with diabetic animals to obtain blood-glucose levels comparable with the normals. In the latter group the diabetic state of the animal was always confirmed by a glucose tolerance test.

The substance under investigation was injected intravenously into the ear vein, at the level of 1 g./kg. (expressed as glucose) as a 10% solution.

Experiments in which both ureters were ligated to prevent excretion of the injected phosphate esters in the urine were carried out as follows. The rabbits were lightly anaesthetized with ether and a median incision was made in the abdominal wall at the level of the bladder; the ureters were rapidly ligated and the wound closed. The operation was carried out about 2 hr. before the injection, and the animals killed immediately at the end of the experiment. The substances tested were injected in half the normal dose (0·5 g./kg., expressed as glucose). Control experiments, in which rabbits were operated on as described above but without injection of test substances, did not show appreciable alterations in the blood glucose during the 6 hr. period following the operation.

In all experiments blood samples were taken immediately before and 3, 15, 30, 60 and 120 min. after injection. About 2·5 ml. of blood were taken from the heart and transferred to a small test tube containing two drops of heparin. A 0·1 ml. sample was taken for glucose analysis, and 2 ml. of blood were added to 2 ml. of 10% trichloroacetic acid and centrifuged. The protein-free filtrate was analysed as described above.

The animals were kept in metabolism cages; in some experiments the urine was collected as excreted over a period of 24 hr. after injection, in other experiments the urine was collected by means of a catheter at regular intervals.

In connexion with the *in vivo* experiments, tests for phosphatase activity in the blood were carried out using the following procedure. Blood was withdrawn from the heart of a normal rabbit and a minimal amount of heparin added to prevent clotting. The blood was immediately mixed with solutions of G1-P or G6-P in the proportion of 2 ml. of blood to 1 ml. of a 5% solution of substrate (expressed as glucose concentration) previously warmed to 37°. Samples were taken for analysis of inorganic P immediately after the addition of blood and after 1 and 2 hr. incubation at 37°. The protein was precipitated with 10% trichloroacetic acid and the inorganic P of the supernatant was determined.

Procedure for in vitro *experiments*

Male rats weighing 120–180 g. were used. All animals were fasted for 24 hr. prior to the experiment. The isolated rat diaphragm technique, first described by Gemmill (1940, 1941), was adopted.

The rats were killed by decapitation. The two hemidiaphragms (weighing approximately 100 mg. each) were rapidly removed and weighed on a torsion balance to the nearest mg. Each hemidiaphragm was then transferred to 3 ml. of medium contained in a Warburg vessel and previously chilled in ice. The medium was at pH 6·8 and identical with that used by Stadie & Zapp (1947), containing 0·04 M-Na_2HPO_4, 0·08 M-NaCl and 0·005 M-$MgCl_2$. The substrate under investigation was used in a final concentration of 2 or 0·2 %.

The flasks were attached to manometers, oxygenated for 5 min. and then incubated for 90 min. at 37°. At the end of the incubation period the flasks were removed from the manometers and immersed in ice.

Glycogen synthesis. Equal parts, approximately one-third of each half of the diaphragm, were taken together, weighed and immediately immersed in 2 ml. of 30% KOH for the determination of the initial glycogen content. The remaining two-thirds of each hemidiaphragm were incubated as described above. After incubation the diaphragms were removed, blotted on filter paper, rapidly washed in a physiological saline solution and transferred to 2 ml. of 30% KOH.

Substrate utilization. Samples of the medium were taken before and after incubation and analysed by the methods described above.

Respiration. The O_2 consumption was measured by the usual Warburg manometric technique.

Insulin effect. An insulin solution (0·1 ml.), containing 3 units, was added to the medium giving a final concentration of 1 unit/ml. Insulin was added to one hemidiaphragm of each rat and the results compared with those for the other hemidiaphragm incubated without insulin.

2:4-*Dinitrophenol effect.* A solution of 2:4-dinitrophenol (DNP) (0·1 ml.) was added to the medium before incubation to give a final concentration of $4{\cdot}83 \times 10^{-5}$ M.

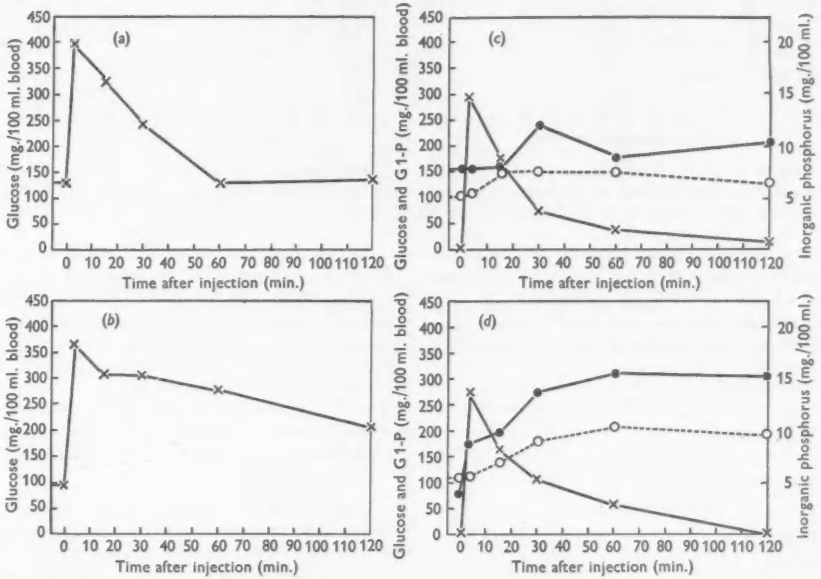
(a)
(b)
(c)
(d)
Glucose (mg./100 ml. blood)
Glucose and G 1-P (mg./100 ml. blood)
Inorganic phosphorus (mg./100 ml.)
Time after injection (min.)
0 10 20 30 40 50 60 70 80 90 100 110 120

Fig. 1. Rate of disappearance of intravenously injected glucose and G1-P from the blood of normal and alloxan-diabetic rabbits with accompanying increase in blood inorganic phosphorus and glucose. 1 g./kg. injected. The alloxan-diabetic rabbits were maintained on a carbohydrate-free diet prior to experiments to reduce the blood glucose to normal. Experiments carried out on the same diabetic rabbit on two consecutive days. (*a*) Normal rabbit (glucose injection). (*b*) Alloxan-diabetic rabbit (glucose injection). (*c*) Normal rabbit (G1-P injection); ○- - -○, glucose; ●—●, inorganic phosphorus; ×—×, G1-P. (*d*) Alloxan-diabetic rabbit (G1-P injection); key as for (*c*).

RESULTS

I. *In vivo* EXPERIMENTS

The metabolism of G 1-P and G 6-P has been studied and compared with that of glucose, in normal and alloxan-diabetic rabbits. Experiments with G 1-P were carried out on five normal and seven diabetic rabbits, and with G 6-P on nine normal and seven diabetic rabbits. Similar results were obtained for all the animals, and typical illustrative examples are given in Figs. 1 and 2.

It was found that both G 1-P and G 6-P, injected intravenously, rapidly disappeared from the blood of both normal and alloxan-diabetic rabbits. In normal rabbits the rate of disappearance from the blood, i.e. the tolerance curve, was similar to that of glucose. In the alloxan-diabetic rabbits the glucose organic phosphorus and glucose levels reached a maximum in most experiments in 30–60 min. In the 24 hr. period following the injections a very considerable increase in the excretion of inorganic phosphorus in the urine of all animals was observed (Fig. 4).

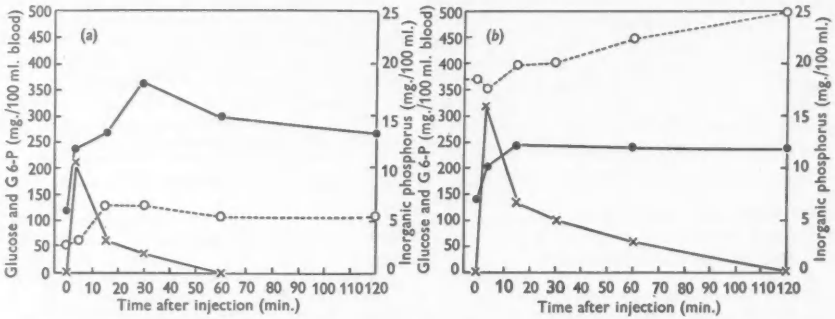

Fig. 2. Rate of disappearance of intravenously injected G6-P from the blood of normal and alloxan-diabetic rabbits with accompanying increase in blood inorganic phosphorus and glucose. 1 g./kg. injected. (*a*) Normal rabbit. (*b*) Alloxan-diabetic rabbit. O- - -O, Glucose; ●—●, inorganic phosphorus; ×—×, glucose 6-phosphate.

level of the blood remained high for a considerable time after injection in striking contrast to G 1-P and G 6-P which disappeared at a normal rate. In three diabetic rabbits, glucose and G 1-P were injected simultaneously; the results of one of these experiments is shown in Fig. 3.

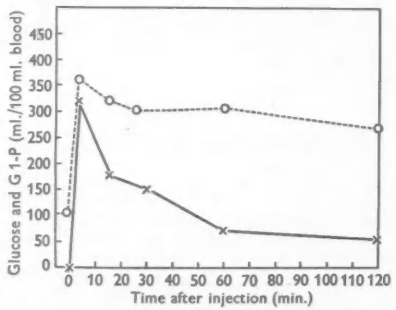

Fig. 3. Rate of disappearance from the blood of glucose and G 1-P injected simultaneously into an alloxan-diabetic rabbit. 1 g./kg. of each substance injected intravenously. O- - -O, Glucose; ×—×, G 1-P.

Following the injection of G 1-P and G 6-P the blood glucose increased from 30 to 100 mg./100 ml. in normal rabbits, and, as was to be expected, more markedly in diabetic rabbits. The inorganic phosphorus of the blood also increased from 4 to 12 mg./100 ml. (Table 1). The results show that there was a marked variation in the increase in blood glucose in different animals. Both the in-

As the results obtained in the above experiments could be explained by a breakdown of the phosphate esters in the blood, *in vitro* experiments were carried out with blood from two rabbits to ascertain whether or not this breakdown occurs. The results given in Table 2 show that no significant amount of either

Table 1. *Maximum increase of inorganic phosphorus and glucose in the blood following intravenous injection of G 1-P and G 6-P in normal and alloxan-diabetic rabbits*

(Injected 1 g./kg. Results expressed as mg./100 ml. of blood. Each line represents the results of an experiment on one rabbit treated with G1-P and one with G6-P.)

G1-P injected		G6-P injected	
Increase in inorganic P	Increase in glucose	Increase in inorganic P	Increase in glucose
	Normal animals		
4·2	47	8·6	—
8·8	60	11·2	—
7·5	70	9·3	92
8·5	108	14·4	44
10·6	35	6·6	27
		5·0	45
		9·0	52
		12·2	55
		9·0	46
	Diabetic animals		
11·6	38	10·3	93
8·5	270	9·2	94
12	90	9·6	195
12	106	7·8	140
11	105	6·8	103
7·6	54	8·0	202
11·6	170	10	145

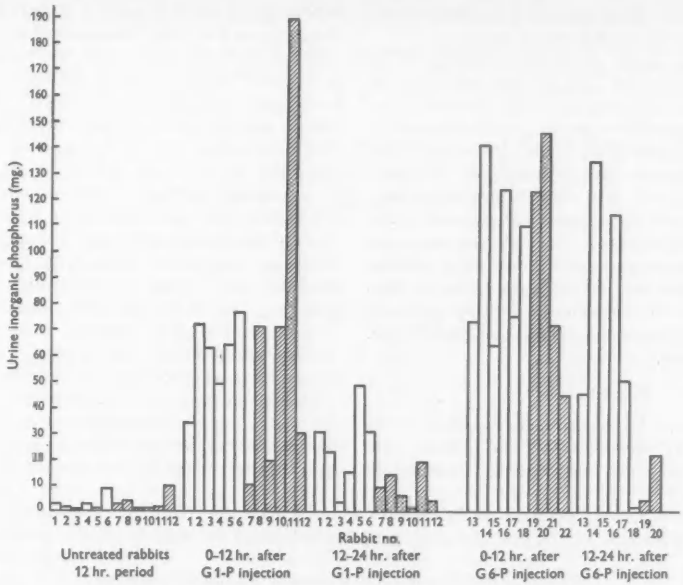
Urine inorganic phosphorus (mg.)
Rabbit no.
Untreated rabbits 12 hr. period
0-12 hr. after G1-P injection
12-24 hr. after G1-P injection
0-12 hr. after G6-P injection
12-24 hr. after G6-P injection

Fig. 4. Excretion of inorganic phosphorus in the urine of normal and alloxan-diabetic rabbits, before and after injection of G1-P or G6-P. ▨, Alloxan-diabetic rabbits; □, normal rabbits.

Table 2. *The phosphatase activity of normal rabbit blood with G1-P and G6-P as substrates*

(The reaction mixtures contained 2 ml. whole blood and 1 ml. of a 5% solution of substrate (expressed as glucose).)

Rabbit	Substrate	Time of incubation at 37° (hr.)	Inorganic P liberated (μg./ml. of substrate)	Substrate hydrolysed (%)
1	G1-P	1	0	0
		2	0	0
	G6-P	1	8·4	—
		2	28·6	0·28
2	G1-P	1	0	—
		2	15	0·17
	G6-P	1	37·2	—
		2	103·2	1·20

Table 3. *Percentage of intravenously injected G1-P and G6-P excreted in the urine of normal and alloxan-diabetic rabbits*

(Injected 1 g./kg. Mean values given ±S.E.M. Number of rabbits and range given in parentheses. Urine collected for 24 hr. following injection.)

G1-P excreted (%)		G6-P excreted (%)	
Normal	Diabetic	Normal	Diabetic
31±3·6	31±3·1	44±6·4	55±4·7
(7)	(7)	(9)	(5)
(14–43)	(22–43)	(25–90)	(44–65)

G1-P or G6-P is hydrolysed when incubated for 2 hr. at 37° with whole blood.

The percentage of injected G1-P and G6-P excreted in the urine of normal and diabetic rabbits during a period of 24 hr. following injection is given in Table 3. These values showed that there was no significant difference between the percentage excreted in the normals and the diabetics, and also that taking the mean values there was no significant difference between the excretion of G1-P and G6-P although in a few rabbits a very high excretion of G6-P was observed.

In order to prevent excretion some experiments were carried out on normal and diabetic rabbits in

which the ureters were ligated 2 hr. before the injection of G 1-P and G 6-P (0·5 g./kg.).

The results of these experiments are given in Table 4 and show that: (1) in most animals the phosphate esters had completely disappeared from the blood 1 hr. after the injection, (2) the increase in inorganic phosphorus of the blood in relation to the amount of phosphate ester injected was, as would be expected, higher than in animals in which a large amount of inorganic phosphorus was excreted in the urine, and (3) the increase in blood glucose was, as in experiments with unoperated animals, very variable but in most cases was of the same order as previously observed. The maximum increase in blood inorganic phosphorus and glucose occurred 1–2 hr. after the injection.

Toxicity tests

In carrying out the experiments reported above it was frequently observed that the animals presented signs of acute intoxication immediately following the injection of G 1-P or G 6-P, which, if the animals survived, disappeared in 5–10 min. It was therefore decided to test the toxicity of these substances when injected intraperitoneally into mice, and to compare their toxicity with an equivalent amount of inorganic phosphate, injected into mice under the same conditions. The results are given in Table 5, and show that there was no significant difference between the toxicity of G 1-P and G 6-P and that their toxicity was only slightly less than that of an equivalent amount of inorganic phosphate. There was, however, a considerably longer delay before the outset of the toxic action of the phosphate esters than after the injection of sodium phosphate. The LD_{50} value at normal temperature was approx. 6·9 mg. P/20 g. mouse for G 1-P, G 6-P and for inorganic phosphate.

It was found in later experiments with rabbits that the toxic manifestations which appeared immediately after injection in some of the first experiments could almost always be avoided by

Table 4. *Concentration of intravenously injected G 1-P and G 6-P in the blood of normal and alloxan-diabetic rabbits with ligated ureters, and the accompanying maximum increase in blood inorganic phosphorus and glucose*

(Results expressed as mg./100 ml. blood. 0·5 g./kg. injected.)

G 1-P injected				G 6-P injected			
Concentration of G 1-P in blood following injection		Increase in inorganic P	Increase in glucose	Concentration of G 6-P in blood following injection		Increase in inorganic P	Increase in glucose
3 min.	60 min.			3 min.	60 min.		
Normal animals							
260	73	16	157	162	0	11	127
240	22	19	107	—	—	—	—
Diabetic animals							
141	13	11	120	100	0	10·2	70
158	0	9·8	50	130	0	9·6	200
—	—	—	—	169	60	11·4	115
—	—	—	—	95	0	10·8	190
—	—	—	—	127	0	11·3	70
—	—	—	—	110	0	10·6	35

Table 5. *Toxicity of G 1-P, G 6-P and sodium phosphate in mice*

(Mice weighing approx. 20 g. were injected intraperitoneally with substances tested. Exps. 1 and 2 carried out at 37 and 18°, respectively.)

Exp.	Dose (mg./20 g. mouse) expressed as Inorganic P	Dose (mg./20 g. mouse) expressed as Glucose	G 6-P No. of deaths/ total no.	G 6-P Times of death (min.)	G 1-P No. of deaths/ total no.	G 1-P Times of death (min.)	Sodium phosphate No. of deaths/ total no.	Sodium phosphate Times of death (min.)
1	3·4	20	0/5	—	0/5	—	0/5	—
	4·3	25	0/5	—	0/5	—	0/5	—
	5·2	30	3/5	100–305	3/5	140–310	5/5	50–80
	6·9	40	5/5	125–150	5/5	80–105	—	—
	8·6	50	5/5	50–110	5/5	60–100	5/5	40–50
2	5·2	30	1/5	110	0/5	—	0/5	—
	8·6	50	4/5	205–265	3/5	80–110	5/5	40–90
	11·2	65	4/5	180–240	5/5	120–240	5/5	40–50
	13·8	80	5/5	70–240	5/5	240–255	—	—

Table 6. *The effect of glucose, G 1-P and G 6-P on insulin convulsions in mice*

(Experiments carried out at 37°. Insulin was injected intraperitoneally as indicated, and as soon as convulsions started the mice were injected intraperitoneally with glucose, G 1-P or G 6-P in the doses indicated, expressed as mg./20 g. mouse. The control groups were untreated. In experiments in which some of the animals survived, the arbitrary time of 180 min. was taken in calculating the mean values.)

		Glucose injections		G 1-P injections		G 6-P injections	
	Controls	25 mg.	50 mg.	25 mg.	50 mg.	25 mg.	50 mg.
	Exp. 1. 0·02 unit insulin/20 g. mouse						
No. of mice	20	10	5	10	5	10	5
Time between convulsions and death (min.). Mean ± S.E.M.	14·5 ±2·5	101·6 ±12·7	97 ±7·0	56·9 ±15·0	37·8 ±4·0	42·3 ±6·2	33·6 ±3·9
No. of animals surviving	0	4	1	1	0	0	0
	Exp. 2. 0·10 unit insulin/20 g. mouse						
No. of mice	20	—	10	—	10	—	10
Time between convulsions and death (min.). Mean ± S.E.M.	13·5 ±2·1	—	99·9 ±13·3	—	36·9 ±5·1	—	29 ±5·8
No. of animals surviving	0	—	2	—	0	—	0

injecting the solutions slowly (about 2 min. for 20 ml.).

Effect of glucose, G 1-P and G 6-P on insulin convulsions in mice

The curative effect of the esters on insulin convulsions in mice was compared with that of glucose. The technique used for these experiments and the results are given in Table 6. Although a 50 mg. dose of glucose/20 g. mouse was insufficient to assure complete protection, this was the maximum dose tested owing to the toxicity of the phosphate esters at higher concentrations; in fact a 50 mg. dose of G 1-P or G 6-P was less effective in prolonging life than a 25 mg. dose.

The findings of Marks & Morgan (1927) that a subcutaneous injection of hexose mono- or diphosphate did not relieve symptoms of insulin convulsions in rabbits or mice can be readily explained in their experiments with mice by the fact that the dose of the phosphate esters injected (100 mg., expressed as glucose, per mouse) is toxic.

Our results show that both G 1-P and G 6-P prolonged the lives of the mice after onset of insulin convulsions, but were less effective than glucose; the reason for this is not clear.

II. *In vitro* EXPERIMENTS WITH ISOLATED RAT DIAPHRAGM MUSCLE

The metabolism of G 1-P

The metabolism of G 1-P by normal rat diaphragm has been compared with that of glucose as regards the uptake of substrate, glycogen formation and effect on respiration. The influence of insulin on the utilization of glucose and G 1-P has also been compared.

Uptake of substrate. The results are given in Table 7. It appeared that the uptake of G 1-P was higher than that of glucose in the absence of insulin, but that insulin caused a highly significant increase in the uptake of glucose (as first observed by Gemmill & Hamman, 1941, and later confirmed by Stadie, 1944; Krahl & Cori, 1947; Perlmutter & Greep, 1948; Krahl & Park, 1948; Villee & Hastings, 1949), and a significant decrease in the disappearance of G 1-P.*

Table 7. *Effect of insulin on utilization of glucose and G 1-P by rat diaphragm muscle*

(Results expressed as mg. of substrate disappearing from medium/g. wet wt. of diaphragm in 90 min. at 37°, in O_2 (mean values ± S.E.M.). Substrates, 0·2%; insulin, 1 unit/ml. of medium. A positive or negative insulin effect indicates an increase or decrease in substrate utilization, respectively.)

		Substrate uptake		
No. of rats	Substrate	No insulin	With insulin	Insulin effect
12	Glucose	4·40* ±0·30	9·73 ±0·69	+5·33 ±0·33 $P<0·01$
10	G 1-P	7·08* ±0·58	4·46 ±0·57	−2·62 ±0·33 $P<0·01$

* In the absence of insulin there is a highly significant difference in the uptake of glucose and G 1-P ($P<0·01$).

Glycogen synthesis. The results given in Table 8 show that the degree of glycogen synthesis both from glucose and G 1-P was largely dependent on the initial concentration of glycogen present in the diaphragm, in accordance with the results of Hansen, Rutter & Samuels (1951). Therefore, the experiments were divided into groups depending on the initial glycogen content. When the initial con-

* Since the completion of this work the authors have observed that part of the G 1-P is converted into F 6-P in the medium.

Table 8. *Effect of insulin on glycogenesis from glucose and G 1-P by rat diaphragm muscle*

(The diaphragms were incubated for 90 min. at 37° in 3 ml. of medium in O_2. Mean values are given ± S.E.M. Substrates, 2% glucose or G 1-P. Insulin 1 unit/ml. of medium. Glycogen synthesis indicates the increase in glycogen over the initial value. A positive or negative insulin effect indicates an increase or decrease in glycogen synthesis respectively.)

No. of rats	Initial glycogen content of diaphragm: Limiting values	Initial glycogen content of diaphragm: Mean values	Substrate	Glycogen synthesis (mg./g. wet wt.) tissue: No insulin	Glycogen synthesis (mg./g. wet wt.) tissue: With insulin	Insulin effect	P
4	0·20–0·39	0·25	G 1-P	0·88±0·20	0·72±0·11	−0·16±0·12	0·85
5	0·40–0·59	0·49	Glucose	0·76±0·14	1·57±0·38	+0·81±0·32	0·10
5	0·40–0·59	0·49	G 1-P	0·72±0·26	0·78±0·25	+0·06±0·04	0·85
8	0·60–0·79	0·69	Glucose	0·65±0·15	1·09±0·14	+0·44±0·09	0·04
6	0·60–0·79	0·66	G 1-P	0·70±0·21	0·86±0·26	+0·16±0·01	0·60
6	0·80–0·99	0·88	Glucose	0·45±0·16	0·60±0·09	+0·15±0·09	0·40
4	0·80–0·99	0·91	G 1-P	0·47±0·07	0·43±0·08	−0·04±0·06	0·70
6	>1·00	1·25	Glucose	0·02±0·18	0·58±0·16	+0·56±0·13	0·05
6	>1·00	1·39	G 1-P	0·12±0·18	0·09±0·19	−0·03±0·06	0·90

Results are considered significant when $P = 0·1$ or less.

centration exceeded 1 mg./g. wet weight of tissue there was usually little or no synthesis of glycogen and in some cases a glycogenolysis was observed.

In accordance with the findings of other authors (Gemmill, 1941; Stadie & Zapp, 1947; Corkill & Nelson, 1947; Perlmutter & Greep, 1948; Villee & Hastings, 1949) in most cases there was a significant increase in glycogen synthesis from glucose in the presence of insulin (Table 8). Only in the group with an initial glycogen content of 0·88 mg./g. (mean value) was no significant insulin effect observed in the presence of glucose. With G 1-P there was no significant effect of insulin in any group. The substrates were used in a concentration of 2%, as at lower concentration there was little or no synthesis of glycogen from G 1-P (Verzár & Wenner, 1948).

Table 9. *Influence of the ionic concentration on the insulin effect on glycogenesis*

(The diaphragms were incubated for 90 min. at 37° in 3 ml. of medium, containing glucose (2%) and 1 unit insulin/ml. Gas phase, O_2. Mean values given ± S.E.M.)

No. of rats	Initial glycogen (mg./g. wet wt.) tissue	NaCl concn. of medium (M)	Increase in glycogen (mg./g. wet wt.) tissue
8	0·69	0·08	0·44* ±0·09
4	0·66	0·20	0·44 ±0·02

* Results taken from Table 8.

In order to ascertain that the difference in the effect of insulin on glycogen synthesis in systems containing glucose and G 1-P, respectively, was not due to the increased ionic concentration of the medium in the latter, some experiments were carried out with glucose in a medium containing an increased sodium chloride concentration (0·20M) so that the ionic conditions closely approximated those of the G 1-P experiments. The results are given in Table 9, and show that the insulin effect is not altered by the increased ionic concentration of the medium. As only four rats were used for these experiments, no statistical analysis of the results was made.

Table 10. *Oxygen consumption by rat diaphragm muscle in the presence of glucose, G 1-P and G 6-P*

(Results expressed as µl. O_2/100 mg. wet weight of diaphragm in 90 min. at 37° in O_2. Mean values given ± S.E.M. Substrates, 2% glucose, G 1-P and G 6-P. The hemidiaphragms from each rat were incubated in glucose and G 1-P respectively, or glucose and G 6-P respectively.)

No. of rats	O_2 consumption (µl.): Glucose	O_2 consumption (µl.): G 1-P	Increase in O_2 consumption in the presence of G 1-P (µl.)
8	209 ±6·6	324 ±17·4	115 ±16·7 $P = 0·01$
	Glucose	G 6-P	
3	230 ±10·0	217 ±14	

It might be suggested that the addition of sodium phosphate to the medium, instead of sodium chloride, would have created ionic conditions more closely resembling those existing in the presence of the sodium salt of the phosphate ester; this, however, was not feasible owing to the inhibitory effect of high concentrations of inorganic phosphate on glycogen synthesis, a phenomenon described in experiments with rat diaphragm muscle by Stadie & Zapp (1947), and confirmed by the present authors.

Oxygen consumption. The results of these experiments are shown in Table 10. There was a considerably higher oxygen consumption by the diaphragm

Table 11. *Effect of 2:4-dinitrophenol (DNP) on oxygen consumption by rat diaphragm muscle in presence of glucose and G 1-P*

(Results expressed as μl. O_2/100 mg. wet wt. of diaphragm/90 min. at 37° in O_2. Mean values given $\pm$S.E.M. Concentration of DNP = $4{\cdot}83 \times 10^{-5}$M. A positive or negative effect of DNP indicates an increase or decrease in O_2 uptake, respectively. The hemidiaphragms from each rat were incubated with and without DNP respectively.)

No. of rats	Substrate (2%)	O_2 uptake (μl.) No DNP	O_2 uptake (μl.) With DNP	Effect of DNP
9	Glucose	170±10	342±13·4	+172±16·6 $P = <0{\cdot}01$
9	G 1-P	273±8·9	168±8·7	−105±12·7 $P = <0{\cdot}01$

muscle incubated with G 1-P compared with that incubated with glucose. The addition of glucose to the medium had no effect on the respiration of diaphragm muscle.

The effect of 2:4-dinitrophenol on respiration. Another interesting contrast in the behaviour of glucose and G 1-P is the effect of DNP on the respiration of rat diaphragm muscle in the presence of these substrates. The results (Table 11) show that in the presence of glucose, in confirmation of the findings of Pierce & Field (1949), the oxygen consumption was greatly increased by DNP, whereas in the presence of G 1-P a considerable inhibition was observed.

The metabolism of G 6-P and F 6-P

Equilibrium between G 6-P and F 6-P. It was shown that in rat diaphragm muscle there is a very active phosphohexoisomerase enzyme, described by Lohmann (1933) in muscle suspensions, and by Wajzer (1951) in haemolysed blood, which acts on F 6-P or G 6-P forming an equilibrium mixture of the two phosphate esters containing approximately 40% F 6-P and 60% G 6-P. The transformation of one ester into the other was measured either by the disappearance of F 6-P when added to the medium, or by the formation of F 6-P when G 6-P was the added substrate (Table 12). The total phosphorus of the medium was not found to alter during incubation, and this suggested that there was an isomerization of one phosphate ester into the other. This was confirmed by paper chromatography according to the method of Hanes & Isherwood (1949).

Table 12. *Isomerization of F 6-P and G 6-P by rat diaphragm muscle*

(Rat diaphragm muscle incubated for 30 min. at 37° in presence of F 6-P or G 6-P. Mean values given $\pm$S.E.M.)

No. of rats	Substrate and concentration	F 6-P disappearing from medium (%)	G 6-P converted into F 6-P (%)
3	2% G 6-P	—	44±0·55
3	2% F 6-P	62±2·65	—
3	0·2% G 6-P	—	47±1·0
3	0·2% F 6-P	60±2·52	—

The phosphohexoisomerase was found to be active at room temperature and the equilibrium was established in about 15 min. The enzyme appeared to diffuse out readily from the muscle. Thus it was found that by washing the diaphragm muscle for 15 min. in 3 ml. of medium, an active extract of the isomerase was obtained, and on repeating this extraction three times with three lots of medium, the isomerase enzyme was active in each extract and the diaphragm itself still retained some activity.

In the following experiments on oxygen consumption and glycogen synthesis, 2% G 6-P was the added substrate; however, owing to the rapid formation of an equilibrium mixture by the isomerase enzyme, the medium in fact contained approximately 0·8% F 6-P and 1·2% G 6-P.

Oxygen consumption. The results of these experiments are given in Table 10, and show that there is no increase in oxygen consumption in the presence of G 6-P as added substrate.

Glycogen synthesis. The results given in Table 13 show that in the absence of insulin a glycogenolysis occurs when diaphragm muscle is incubated in an equilibrium mixture of G 6-P and F 6-P. In the presence of insulin glycogenolysis was inhibited in most experiments, and in some a little glycogen was synthesized.

The metabolism of fructosediphosphate

As shown in Table 13, there was no synthesis of glycogen in the presence of 2% F 1:6-P. No direct comparison was made of the respiration in the presence of glucose and F 1:6-P with hemidiaphragms of the same rats; however the oxygen uptake observed in the presence of 2% F 1:6-P was of the same order as that found in other experiments with 2% glucose as substrate.

DISCUSSION

In vivo *experiments.* The results reported in the present paper bring out two main points. First, contrary to what has been suggested in the literature

Table 13. *Change in glycogen content of rat diaphragm muscle incubated with G 6-P with and without insulin or F 1:6-P without insulin*

(The diaphragms were incubated for 90 min. at 37° in 3 ml. of medium in O_2. Mean values given ±S.E.M. Substrates, 2%. Insulin, 1 unit/ml. medium. A negative change indicates glycogenolysis.)

No. of rats	Initial glycogen content (mg./g. wet wt.) diaphragm: Limiting values	Initial glycogen content (mg./g. wet wt.) diaphragm: Mean values	Added substrate	Change in glycogen content (mg./g. wet wt.): No insulin	Change in glycogen content (mg./g. wet wt.): With insulin	Insulin effect	*P*
8	0·20–0·59	0·32	G 6-P	−0·02±0·01	+0·19±0·01	0·21±0·01	0·10
8	0·60–1·00	0·82	G 6-P	−0·07±0·09	+0·22±0·06	0·29±0·10	0·10
9	1	1·32	G 6-P	−0·12±0·05	+0·07±0·07	0·19±0·10	0·02
4	0·4–0·8	0·66	F 1:6-P	−0·44±0·10	—	—	—

(Conway, 1942; Verzár & Wenner, 1948), the hexose phosphates can penetrate into the tissue cells and are metabolized. The rapid disappearance of intravenously injected G 1-P and G 6-P from the blood, which cannot be accounted for by a phosphatase activity in the blood or by a quantitative excretion of the esters in the urine, can only be explained by the passage of the esters from the blood and extracellular spaces into the tissue cells. The subsequent increases in blood glucose and inorganic phosphorus in the blood and urine supply strong evidence that the esters are metabolized in the cells, although no evidence is available as to which tissues are concerned.

It is impossible to estimate from our results what proportion of the injected phosphate esters are acted on by the tissue phosphatases. However, if the sole metabolic pathway of the esters is the hydrolysis to glucose and inorganic phosphorus, a consistently greater increase in the blood glucose of the diabetic rabbits, particularly in those experiments with animals in which the ureters were ligated, might be expected.

The work of Roberts & Wolffe (1951) is of interest as regards the question of permeability of the hexose phosphate esters into living cells. Using F 6-P and F 1:6-P labelled with ^{32}P, these authors report that the cells of *Escherichia coli* are readily permeable to these esters.

The findings that about 30% of the G 1-P and 40–50% of the G 6-P injected are excreted in the urine are of interest in so far as they indicate that the two phosphate esters must be transferred intact through the glomerular membrane, or less likely through the tubules.

The second main point of interest arising from the *in vivo* experiments is that both G 6-P and G 1-P, in contrast to glucose, appear to have a normal tolerance curve in the alloxan-diabetic rabbit, and excretion of these esters was essentially the same in normal and diabetic animals. These findings can be interpreted as additional evidence for the hypothesis that at least one block in carbohydrate metabolism in the diabetic animal occurs in the phosphorylation of glucose.

In vitro experiments. The first main observation of interest arising from these experiments with rat diaphragm muscle was the absence of a positive insulin effect on substrate uptake and glycogen synthesis in the presence of G 1-P in contrast to that observed in the presence of glucose. This observation is consistent with the theory that insulin is concerned with a preliminary phosphorylation of glucose. This theory, however, cannot account for the apparently inhibitory effect of insulin on G 1-P uptake and the authors at present have no explanation for this effect. The increased oxygen consumption by rat diaphragm muscle in the presence of G 1-P, and the action of DNP on the systems studied, can also not be explained at present.

The second important result in these experiments was the fundamental difference observed between the behaviour of G 1-P and G 6-P in the presence of rat diaphragm muscle. G 6-P was shown to form rapidly an equilibrium mixture with F 6-P, and diaphragm muscle incubated in this mixture neither synthesized glycogen nor showed an increase in oxygen consumption. In fact, G 6-P was found to be relatively inert in striking contrast to G 1-P. These observations are not consistent with the generally accepted theories of glucose metabolism, in which it is assumed that G 6-P is always the first intermediate.

SUMMARY

1. It has been demonstrated that the hexose phosphates can penetrate into the tissue cells of rabbits, and evidence is advanced that they are metabolized within the cells.

2. In contrast to the behaviour of glucose, intravenously injected glucose 1-phosphate and glucose 6-phosphate disappeared rapidly from the blood of both normal and alloxan-diabetic rabbits; a subsequent increase in blood glucose and in the inorganic phosphorus of the blood and urine was observed.

3. Approximately 30% of the injected glucose 1-phosphate was recovered in the urine of both normal and alloxan-diabetic rabbits and 40 and 55% of the injected glucose 6-phosphate in the urine of normal and diabetic rabbits, respectively.

4. In the presence of isolated rat diaphragm muscle, the uptake of glucose 1-phosphate from the medium was higher than that of glucose. Addition of insulin increased the glucose uptake and inhibited the uptake of glucose 1-phosphate.

5. The amount of glycogen synthesis by rat diaphragm muscle was of the same order in systems containing glucose 1-phosphate (2%) and systems containing glucose (2%), and in both cases was largely dependent on the initial glycogen content of the muscle. Insulin increased the glycogen synthesis from glucose but had no effect on glycogen synthesis from glucose 1-phosphate.

6. Oxygen consumption of isolated rat diaphragm was increased by the addition of glucose 1-phosphate (2%) and unaffected by glucose. When 2:4-dinitrophenol ($4{\cdot}83 \times 10^{-5}$ M) was added to the medium, oxygen consumption was increased in the presence of glucose and inhibited in the presence of glucose 1-phosphate.

7. Glucose 6-phosphate and fructose 6-phosphate were acted on by an isomerase enzyme present in rat diaphragm muscle forming an equilibrium mixture containing approximately 40% fructose 6-phosphate and 60% glucose 6-phosphate. Rat diaphragm muscle incubated in this equilibrium mixture did not synthesize glycogen nor was its oxygen consumption affected.

We are indebted to Burroughs Wellcome and Co. for the supply of crystalline insulin used throughout this work.

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Inhibition by Malonate of Succinic Dehydrogenase in Heart-muscle Preparations

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Studies of enzyme inhibition and of the action of enzymes on a variety of substrates can lead to information which may be valuable in the elucidation of the mechanism of enzyme action, since the results indicate a series of molecular structures with which the enzyme combines and thus lead to some conception of the nature of the enzyme active centre.

If a compound is recognized as being a reversible competitive inhibitor, it becomes necessary to express its action in terms of some constant, in order that a series of such inhibitors may be arranged according to potency on some rational and quantitative basis. The most satisfactory method of expressing the potency of a reversible competitive inhibitor is in terms of the dissociation constant of the enzyme-inhibitor complex (K_i), rather than as a degree of inhibition at arbitrarily chosen concentrations of substrate and inhibitor.

As a preliminary to an investigation of this nature to be carried out on succinic dehydrogenase it was felt to be desirable to study the inhibitory action of malonate. The inhibitory action of malonate on succinic dehydrogenase was recognized as being competitive by Quastel & Wooldridge (1928), and it has since become a classical example of a structural analogue acting as a competitive inhibitor. The literature, however, reveals surprising disagreement on the inhibitory power of this substance. The relative affinity of succinic dehydrogenase for malonate has been given values as different as 50 (Potter & DuBois, 1943), 13 (Keilin & Hartree, 1949), 10 (Krebs & Johnson, 1948) and 60 (Krebs, Gurin & Eggleston, 1952). The enzyme preparations used were rat-liver homogenate, heart-muscle preparation, minced pigeon-breast muscle and a yeast preparation, respectively. In some preliminary experiments reported here, a relative affinity as low as 4·7 was obtained. The reason for these discrepancies probably lies in the fact that different types of enzyme preparation were used, and different methods employed for measuring their activity. Slater & Bonner (1952) have shown that in the case of succinic dehydrogenase K_m (the Michaelis constant) cannot simply be regarded as the dissociation constant of the enzyme-substrate complex, but that it corresponds to Briggs & Haldane's (1925) expression $(k_2+k_3')/k_1$, where k_1, k_2 and k_3' are the velocity constants indicated:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3'} E+\text{products}.$$

Since k_3' is larger than k_2, and is dependent on the characteristics of individual enzyme preparations, and on the method of measuring their activity (Slater & Bonner, 1952), K_m determined by conventional methods (Lineweaver & Burk, 1934) is not a constant, and consequently affinity ratios (K_m/K_i) are also variable.

This paper deals with preliminary attempts to determine the K_m/K_i ratio by a direct method in the case of succinate and malonate, with determinations of K_m and K_i separately in a number of preparations, and with the use of malonate in further experiments to determine the velocity constants k_1, k_2 and k_3' by the method described by Slater & Bonner (1952). In the course of this work, some of the discrepancies in the K_m/K_i ratio pointed out above became intelligible in the light of the theoretical explanation outlined (as was predicted by Slater & Bonner (1952)). Furthermore, it is apparent that the limit of K_m, as k_3' approaches zero, is k_2/k_1, the dissociation constant of the enzyme-substrate complex. Comparison of this quantity with K_i should give a true ratio of affinities of succinic dehydrogenase for malonate and succinate.

A preliminary account of this work has already appeared (Thorn, 1953).

METHODS

Enzyme preparation. The preparation was made from pig-heart muscle by the methods of Keilin & Hartree (1947) and Slater (1949*a*). Fat-free dry weights were determined according to Slater (1949*a*), and the phosphate content of the preparations was determined by estimating inorganic phosphate colorimetrically in the trichloroacetic acid supernatant liquids from the dry-weight determinations.

Measurement of succinic dehydrogenase activity. Two main methods were employed, namely, manometric and absorptiometric methods.

(i) Manometric methods. (*a*) Warburg manometers were used, the temperature being 37° and the gas phase, air. Phosphate buffer, pH 7·3, succinate, malonate and KCN, all adjusted to pH 7·3, and methylene blue were contained in

the main compartments of the flasks, and the enzyme was added from the side arms after temperature equilibration. Total fluid volume was 2·5 ml. Final concentrations were: phosphate, 0·15 M; KCN, 0·001 M; methylene blue, 0·001 M; succinate and malonate as shown. The rate of O_2 consumption for the first 20 min. after addition of enzyme was sensibly constant, and was used as the measure of reaction velocity.

(*b*) A similar manometric method was used, but in order to eliminate the effect of competitive inhibition by phosphate (Slater & Bonner, 1952) a borate buffer, pH 7·3, was used instead. The concentration of inorganic phosphate in the reaction mixture, calculated from the amount of inorganic phosphate introduced with the enzyme preparation, was always of the order of $1·7 \times 10^{-3}$ M, and was sufficiently small compared with the K_i of phosphate to have a negligible effect on the determined K_m of the enzyme. Histidine (Bonner, 1951) was also added in order to produce a standard activation of the enzyme in the presence of variable small quantities of phosphate introduced with the enzyme preparation. Total fluid volume was 3·5 ml. Final concentrations were: borate, 0·1 M; histidine, pH 7·3, $8·1 \times 10^{-3}$ M; KCN, 0·01 M; succinate, malonate and methylene blue as shown.

(ii) Absorptiometric method. The rate of reduction of potassium ferricyanide was measured essentially as described by Slater & Bonner (1952), except that the Spekker absorptiometer with the Ilford 601 violet filter was used in place of a photoelectric spectrophotometer set at 400 mμ. All the constituents of the reaction mixture, except the enzyme, were pipetted into glass-stoppered tubes. Enzyme was added to a tube at zero time, and the contents well mixed and tipped into a 1 cm. cell provided with a glass plate as a lid. The temperature of the cell contents was taken after about 1 min. and the cell was placed in the absorptiometer. After readings had been taken until about 5 min. after addition of enzyme, the cell was removed from the instrument and the temperature of its contents again taken. The average rate of change of optical density between 1 and 5 min. after addition of enzyme was used for the calculation of enzyme activities. If precautions were taken to prevent a rise in temperature in the vicinity of the instrument, as a result of the heat of the lamp, the temperature change in the cell during the 4 min. reaction period was not usually more than 0·3°, and the individual runs of an experiment did not differ by more than 0·5° from the average temperature for the whole experiment. Temperature corrections were not applied. The temperatures in this series of experiments lay between 23 and 27·5°, and are stated in the text. The blank cell contained all reagents (including enzyme) except succinate, and with it the instrument was set to a suitable drum reading, usually 0·15, in order to make use of the most convenient part of the scale. Total fluid volume was 7 ml. Final concentrations were: $K_3Fe(CN)_6$, 0·001 M; borate, KCN and histidine as in manometric method (*b*); succinate and malonate as shown.

Measurement of succinic oxidase activity. A manometric method was employed. The details of the method were exactly as described in the manometric method (*b*) for succinic dehydrogenase, except that water was added in place of KCN and methylene blue.

Reagents. Glass-redistilled water was used in making up all solutions.

Succinate. Solutions of succinate were prepared from succinic acid (A.R. grade) and neutralized to pH 7·3 with NaOH.

Malonate. Solutions of malonate were prepared from malonic acid (m.p. 133·5–135° decomp.) and neutralized to pH 7·3 with NaOH.

Phosphate buffers. These were mixtures of KH_2PO_4 and Na_2HPO_4 (both A.R. grade).

Histidine. Solutions of histidine were prepared from L-histidine monohydrochloride M.B.T. (British Drug Houses Ltd.) and were neutralized to pH 7·3 with NaOH.

$K_3Fe(CN)_6$. Solutions of $K_3Fe(CN)_6$ were prepared from the A.R. grade salt, which was recrystallized before use.

The pH of solutions was determined using a glass electrode.

THEORETICAL

The competitive inhibition of succinic dehydrogenase by malonate may be represented by the following scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3'} E + \text{products}, \quad K_m = \frac{k_2 + k_3'}{k_1},$$

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI, \quad K_i = \frac{k_5}{k_4},$$

where E is succinic dehydrogenase, ES is the enzyme-succinate complex, and EI the inactive enzyme-malonate complex. All the methods used are based on the standard Michaelis-Menten expression for the velocity of an enzyme reaction in the presence of a competitive inhibitor, which can be derived from this scheme, namely

$$v = \frac{Vx}{x + K_m(1 + i/K_i)}, \tag{1}$$

where x and i are the concentrations of succinate and malonate respectively, V is the velocity of the reaction at infinite substrate concentration, and v the observed velocity.

Method 1. *Determination of* K_m *and* K_i

K_m and K_i were determined by the methods of Lineweaver & Burk (1934), the basis of which may be simply derived by inverting equation (1). From these values can be obtained the ratio K_m/K_i. This will be described as the 'calculated' value for the ratio, to distinguish it from the directly determined value of the ratio which was obtained by one of the two methods described below.

Method 2. *Determination of the ratio* K_m/K_i *directly*

Equation (1) may be rearranged to the form

$$\frac{V}{v} = 1 + \frac{K_m}{x}\left(1 + \frac{i}{K_i}\right). \tag{2}$$

This equation corresponds to equation (11) of Slater & Bonner (1952), except that it refers to the case of a single competitive inhibitor instead of to the more complex system studied by these workers.

The right-hand side of the equation can be multiplied out to give

$$\frac{V}{v}=1+\frac{K_m}{x}+\frac{K_m}{K_i}\frac{i}{x}, \qquad (3)$$

from which it can be seen that if x is maintained constant and i is varied from 0 to any suitable value, a plot of V/v against i will give a straight line with intercept $1+K_m/x$, and slope $K_m/K_i x$. From the slope of the line, and the given value of x, the desired ratio can be determined.

Method 3. *Approximate determination of the ratio* K_m/K_i

If equation (3) is divided by the expression $1+K_m/x$, the following equation is obtained

$$\frac{V}{v}\left(\frac{x}{K_m+x}\right)=1+\frac{K_m}{K_i}\frac{i}{x}\left(\frac{x}{K_m+x}\right).$$

On the left-hand side of the equation, the product of V and $\left(\frac{x}{K_m+x}\right)$ is the rate of the enzyme reaction at substrate concentration x in the absence of inhibitor. This rate will be designated v_0. The equation may then be rearranged to give:

$$\left(\frac{v_0}{v}-1\right)\frac{x}{i}=\frac{K_m}{K_i}\left(\frac{x}{K_m+x}\right). \qquad (4)$$

This means that, provided the substrate concentration is sufficiently large to make the factor $\left(\frac{x}{K_m+x}\right)$ approximately unity, the left-hand side of the equation equals the desired ratio. An estimate of the ratio can thus be obtained from the degree of inhibition of the reaction at one concentration of substrate and inhibitor. It can be seen from the equation that if the reaction rate is reduced to one-half by the presence of the inhibitor, the left-hand side of the equation becomes x/i, demonstrating the well known fact that the ratio of affinities is equal to the ratio of the concentrations of substrate to inhibitor at 50% inhibition. Equation (4) is equivalent to the formula given by Krebs *et al.* (1952) with their proviso that virtually all the enzyme must be present either in combination with substrate or inhibitor.

RESULTS

Preliminary experiments

In this set of experiments the manometric method (*a*) was used for estimating succinic dehydrogenase activity. Since separate determinations of K_m and K_i involved measurement of rates of oxygen consumption at low substrate concentrations, at which the rate of reaction fell after about 5 min., the main work was directed to determining the ratio K_m/K_i directly, which can be done under conditions where the rate of reaction remains constant for a convenient length of time. One determination of K_m by the method of Lineweaver & Burk (1934) was carried out, however, and gave a value of 5×10^{-4} M. (Since the reaction mixture contained 0·15 M-phosphate, this value must be corrected for the effect of the competitive inhibition of phosphate (Slater & Bonner, 1952). The corrected value of K_m thus becomes 2×10^{-4} M.) No significance is attached to this value, but it provided an approximate figure for calculating V in the following experiments on the direct determination of K_m/K_i.

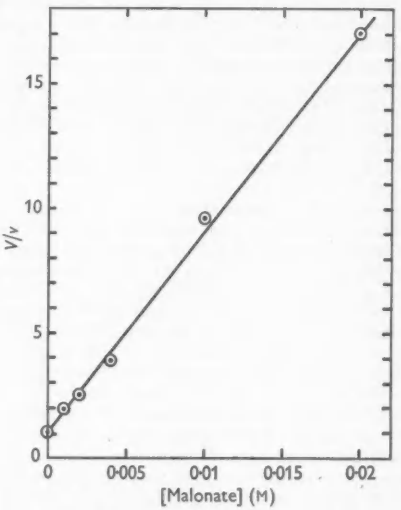

Fig. 1. Effect of malonate concentration on inhibition of succinic dehydrogenase in the presence of a constant amount of succinate. V = maximum velocity; v = observed velocity in the presence of the concentration of malonate shown. Succinic dehydrogenase activity measured manometrically at 37° in the presence of phosphate, pH 7·3, 0·15 M; succinate, 0·01 M; malonate as shown; methylene blue, 0·001 M; KCN, 0·001 M, heart-muscle preparation, approx. 0·6 mg./ml.

A number of experiments was performed to determine the ratio K_m/K_i directly. The kinetic basis of these experiments is given in the Theoretical section, Method 2. One such experiment is illustrated in Fig. 1, where the concentrations of succinate, malonate and methylene blue are given. From the slope of the line and the succinate concentration, the ratio K_m/K_i works out at about 8. (This method gives results which do not require correction for the effect of competitive inhibition by phosphate, since the magnitude of the intercept, not that of the slope, is affected.) The average value obtained for the ratio K_m/K_i, using 0·001 M-

methylene blue, was 6. However, the most significant observation was that the value of the ratio varied with methylene-blue concentration. An experiment illustrating this point is shown in Table 1. The kinetic basis of the calculations is given in the Theoretical section, Method 3. The effect of methylene-blue concentration was shown more fully at a later stage in the investigation.

Table 1. *Effect of methylene-blue concentration on the relative affinity of succinic dehydrogenase for malonate and succinate* (K_m/K_i)

(Activity of succinic dehydrogenase measured manometrically at 37°. All flasks contained phosphate buffer, pH 7·3, 0·15 M; succinate, 0·025 M; KCN, 0·001 M; heart-muscle preparation, 0·67 mg./ml.; malonate (0·01 M) and methylene blue where shown. Total volume 2·5 ml. $\frac{K_m}{K_i} = \left(\frac{v_0}{v} - 1\right)\frac{[\text{succinate}]}{[\text{malonate}]}$, see Theoretical section, Method 3.)

[Methylene blue] ($M \times 10^4$)	Succinic dehydrogenase activity (μl. O_2/hr.): Without malonate (v_0)	With malonate (v)	% Inhibition	K_m/K_i
4	272	94	65	4·7
8	338	102	70	5·8
10	370	105	72	7·5

Determination of K_m and K_i

In these experiments the absorptiometric method of estimating succinic dehydrogenase activity, using potassium ferricyanide as electron acceptor, was employed. This method gives better estimates of initial velocity at low substrate concentrations than does the manometric method. One experiment, demonstrating the effect of substrate concentration on the rate of the reaction in the presence and absence of malonate, is shown in Fig. 2. The results of a number of determinations are given in Table 2, together with the temperatures at which the experiments were carried out, and the calculated K_m/K_i ratios.

The range of values for K_m is from $2·5 \times 10^{-4}$ M to $5·3 \times 10^{-4}$ M, and is to be compared with a figure of $2·3 \times 10^{-4}$ M reported by Slater & Bonner (1952) under similar experimental conditions. The range of values of K_i is from $5·4 \times 10^{-6}$ M to $9·8 \times 10^{-6}$ M. The

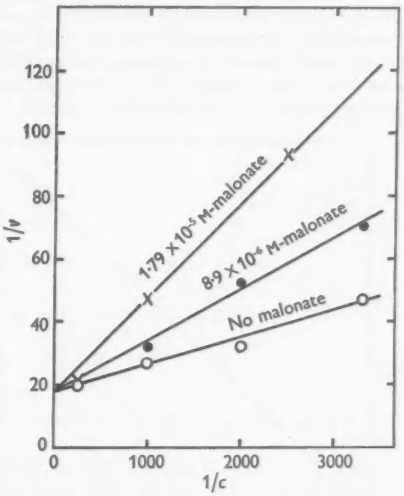

Fig. 2. Competitive inhibition of succinic dehydrogenase by malonate, plotted according to Lineweaver & Burk (1934). c = Molar concentration of succinate; v = change in Spekker reading/min. (Ilford 601 filter). Succinic dehydrogenase activity measured absorptiometrically at 25·3–25·7° in the presence of borate, pH 7·3, 0·1 M; histidine, $8·1 \times 10^{-3}$ M; $K_3Fe(CN)_6$, 0·001 M; KCN, 0·01 M; heart-muscle preparation, 0·42 mg./ml.

spread of the two sets of results is similar and must be attributed at present to experimental error. It might be anticipated that K_m values of different preparations would be different, owing to the complex nature of the reaction and to the participation of a possibly variable quantity, k'_3, in K_m (Slater & Bonner, 1952), but K_i would not be expected to vary,

Table 2. *Values of K_m of succinic dehydrogenase with succinate, and K_i of malonate*

(Succinic dehydrogenase activity measured absorptiometrically with $K_3Fe(CN)_6$ in the presence of cyanide.)

Enzyme preparation	Experiment	Temperature (°)	K_m ($\times 10^4$ M)	K_i ($\times 10^6$ M)	K_m/K_i (calc.)
9	13F	24·8	3·3	9·4	35
	14F	25·5	2·5	—	—
10	16F	24·7	2·8	6·0	47
	17F	23·1	2·7	5·4	50
11	18F	24·5	2·9	6·2	47
	20F	26·9	3·2	9·8	33
13	24F	24·4	5·3	—	—
14	27F	25·6	5·1	9·0	57
15	29F	24·1	5·1	—	—

unless malonate behaves in some anomalous manner. Experiments described below, however, suggest that malonate does in fact behave in a truly competitive manner. The K_m and K_i values shown in Table 2 do not vary consistently with temperature; variation in temperature cannot, therefore, account for the spread of the results. In view of the spread of the results, and the uncertainty in the exact mechanism of participation of potassium ferricyanide in the reaction (Slater, 1949*b*; Tsou, 1951), too much reliance should not be placed on these figures.

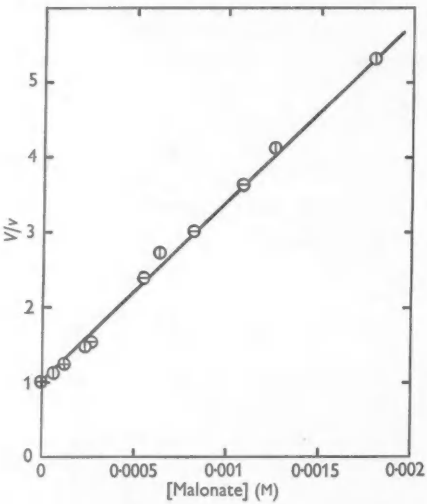

Fig. 3. Effect of malonate concentration on inhibition of succinic dehydrogenase in the presence of a constant amount of succinate. V = maximum velocity; v = observed velocity in the presence of the concentration of malonate shown. Succinic dehydrogenase activity measured absorptiometrically at 27·1–27·4° (ⓘ), and at 26·5–26·7° (⊖), in the presence of borate, pH 7·3, 0·1 M; histidine, $8{\cdot}1 \times 10^{-3}$ M; succinate, 0·025 M; $K_3Fe(CN)_6$, 0·001 M; KCN, 0·01 M; heart-muscle preparation, 0·32 mg./ml.

Further experiments on competitive inhibition by malonate

The absorptiometric method of measuring succinic dehydrogenase activity was used to determine the K_m/K_i ratio directly. The kinetic basis is that given in the Theoretical section, Method 2. The experimental points in Fig. 3 are from two experiments in which malonate concentration was varied at constant succinate concentration. The slope of the line and the succinate concentration give a K_m/K_i ratio of 60, which is to be compared with a calculated ratio of 57 from Exp. 27F in Table 2 since these experiments were carried out with the same preparation. Furthermore, the fact that a straight line is obtained shows that the inhibition involves one molecule of malonate, as might be expected.

The effect of enzyme concentration on inhibition by a given concentration of malonate is shown in Fig. 4. This was a manometric experiment carried out on the complete succinic oxidase system. The points deviate somewhat from linearity, but not to an extent which would suggest that malonate behaved in a 'pseudo-irreversible' manner (Ackermann & Potter, 1949).

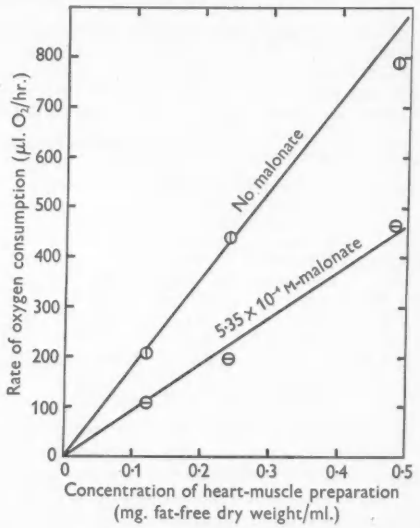

Fig. 4. Effect of enzyme concentration on inhibition of succinic oxidase system by malonate. Succinic oxidase activity measured manometrically at 37° in the presence of borate, pH 7·3, 0·1 M; histidine, $8{\cdot}1 \times 10^{-3}$ M; succinate 0·025 M; malonate as shown.

Determination of the constants k_1, k_2 and k_3'

The constants k_1, k_2 and k_3' were determined exactly as described by Slater & Bonner (1952), except that malonate was used as competitive inhibitor in place of phosphate and fluoride. The reaction medium contained borate buffer and histidine for the reasons given in the Methods section. One experiment is set out in Table 3, which corresponds to Table 6 in the paper by Slater & Bonner (1952).

If the data of Table 3, and similar data from an experiment with preparation 14, are treated exactly as described by Slater & Bonner (1952), values of k_2e and $k_3'e$ (where e is the enzyme concentration) are obtained. From these values, and the relevant figures for K_m shown in Table 2, k_1e can also be calculated. Making the same assumptions as to

Table 3. *Inhibition of succinic dehydrogenase and of the complete succinic oxidase system by malonate*

(Both activities measured manometrically at 37°. Borate, pH 7·3, 0·1 M; succinate, 0·025 M; histidine, $8{\cdot}1 \times 10^{-3}$ M; KCN, where succinic dehydrogenase activity was measured, 0·01 M; malonate ($5{\cdot}36 \times 10^{-4}$ M) and methylene blue where shown; heart-muscle preparation, 0·24 mg./ml. Total volume 3·5 ml. $V = v_0 \left(\frac{K_m + 0{\cdot}025}{0{\cdot}025}\right)$, $K_m = 5{\cdot}1 \times 10^{-4}$ with succinic oxidase system, $5{\cdot}1 \times 10^{-4} \times \frac{v_0}{2 \times 404}$ with succinic dehydrogenase.)

Enzyme activity measured	[Methylene blue] (M × 10⁴)	Activity (μl. O_2/hr.) Without malonate (v_0)	With malonate (v)	% inhibition	V	V/v
Succinic dehydrogenase	1·5	107	90	15·9	107	1·19
Succinic dehydrogenase	3·0	131	107	18·3	132	1·23
Succinic dehydrogenase	10	222	167	24·8	223	1·28
Succinic dehydrogenase	30	257	183	28·8	258	1·41
Succinic oxidase system	—	404	192	52·6	412	2·15

Table 4. *Values of k_1, k_2 and k'_3 for succinic oxidase*

Preparation	Q_{O_2}	Temperature (°)	k_1 (l.mole^{-1} sec.$^{-1}$)	k_2 (sec.$^{-1}$)	k'_3 (sec.$^{-1}$)
14	510	37	$3{\cdot}5 \times 10^4$	1·1	17
15	490	37	$3{\cdot}2 \times 10^4$	0·88	15
Slater & Bonner (1952)	950	38	7×10^4	2	32

enzyme concentration as these workers, the approximate values of k_1, k_2 and k'_3 given in Table 4 were found. It can be seen at once that there is virtually no difference between the results with preparations 14 and 15, and that these figures, together with the Q_{O_2} values, are about half those of Slater & Bonner (1952).

It should be noted that in these calculations the figure for K_m of succinic oxidase at 37° was taken as $5{\cdot}1 \times 10^{-4}$ M. This is in fact the value for the K_m of succinic dehydrogenase at about 25°, with potassium ferricyanide as hydrogen acceptor (see Table 2, preparations 14 and 15). This value was used because it was not possible to determine K_m for succinic oxidase accurately, since only the manometric method was available, and this method was not reliable for determining rates of oxygen consumption at low substrate concentrations. (The published figure for K_m of the succinic oxidase system (Slater & Bonner, 1952), obtained by ultraviolet spectrophotometry, is $4{\cdot}8 \times 10^{-4}$ M at 25°, which is similar to the figure which has been used above.) In any event, since k_2 and k'_3 are derived directly from the experimental data, only k_1 would be greatly affected by errors in the value of K_m, and the striking difference between k_2 and k'_3 is the most interesting result.

In view of the indirect methods used in determining k_1, k_2 and k'_3, and the assumptions involved (as to enzyme concentration and the value of K_m for succinic oxidase at 37°), the agreement between the results reported here and those of Slater & Bonner (1952) is considered good. In fact, the differences between the absolute values for k_1, k_2 and k'_3 obtained in this paper and by Slater & Bonner (1952) can be attributed to the questionable assumption that the concentration of succinic dehydrogenase (per mg. fat-free dry weight) was the same in all preparations, although the Q_{O_2} values differed by a factor of 2. If, on the other hand, it is assumed that the concentration of succinic dehydrogenase is proportional to the Q_{O_2}, the differences between the two sets of results disappear, and the values should then be regarded, not as absolute, but, more correctly, as indications of the order of magnitude of the constants. The most significant part of the results is that the relative magnitudes of k_1, k_2 and k'_3 are in both cases virtually identical; in particular, that the rate of breakdown of the enzyme-substrate complex to give products is sixteen times faster than its rate of breakdown to give the unchanged substrate.

An estimate of the ratio of the dissociation constants of the enzyme-succinate and enzyme-malonate complexes

It has been pointed out in the Introduction that the limit of K_m as k'_3 approaches zero is k_2/k_1. It follows that K_m/K_i ratios determined under conditions of successively decreasing maximum velocities ($V = k'_3 e$) should tend towards the ratio of the dissociation constants of the enzyme-succinate and enzyme-malonate complexes, or conversely, towards what might be described as the true ratio of affinities of succinic dehydrogenase for malonate and succinate. A method of varying V (and hence k'_3) is to use different methylene-blue concentrations. Data such as those in Table 3 may therefore be used

in this extrapolation to zero k_3'. Since the succinate concentration is much greater than K_m, it is possible to work out the ratio K_m/K_i for succinic dehydrogenase at each methylene-blue concentration, and for succinic oxidase (see Theoretical section, Method 3). In Fig. 5 the values of the ratio are plotted against the corresponding values of Q_{O_2}, where the Q_{O_2} has been calculated from the maximum velocity V in Table 3, and is hence directly proportional to k_3'. The points originate from experiments with preparations 14 and 15, and it can be seen that they fall close to a straight line whose

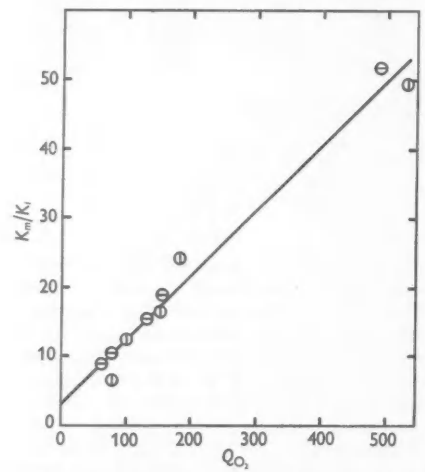

Fig. 5. Variation of relative affinity of succinic dehydrogenase for malonate and succinate (K_m/K_i) with Q_{O_2} (calculated from maximum velocity, V). From data of Table 3 (⊖), and similar data of another experiment (⊕).

ordinate intercept is about 3. This implies that in the absence of further reaction (zero Q_{O_2}, hence zero k_3', and $K_m = k_2/k_1$ = dissociation constant of enzyme-substrate complex) the ratio of affinities of succinic dehydrogenase for malonate and succinate is 3.

DISCUSSION

The inhibition of succinic dehydrogenase by malonate has long been known to be of the reversible competitive type, and the present work was originally intended to determine the relative affinity of succinic dehydrogenase for malonate and succinate. This object implies the comparison of the dissociation constants of the enzyme-succinate and enzyme-malonate complexes. It has been pointed out that there are considerable discrepancies in the various estimates of the ratio of these quantities. The discovery of Slater & Bonner (1952) that K_m for succinic dehydrogenase is not the dissociation constant of the enzyme-succinate complex (k_2/k_1) but the fraction $(k_2+k_3')/k_1$, and that normally k_3' is considerably greater than k_2, but can vary according to the experimental conditions chosen, suggested a better understanding of the problem.

It is clear from the experiments reported here that there is proportionality between k_3' and the ratio K_m/K_i. The extrapolated value of K_m/K_i when k_3' is zero appears to be the true ratio of affinities of the enzyme for malonate and succinate. The extrapolated value of K_m/K_i is about 3, implying that the affinity of the enzyme for malonate is about three times its affinity for succinate. The magnitude of this ratio of affinities is in marked contrast to the figures of 50 (Potter & DuBois, 1943), 60 (Krebs *et al.* 1952) and similar high values recorded under some conditions in this paper. These findings, of course, do not in any way alter the fact that the various relative affinities previously reported in the literature are the experimentally important quantities relevant to the types of preparation in which they were determined. The comparatively small relative affinity of 3 given here is of theoretical interest, and the work reported is a demonstration of the different view which has to be taken of the kinetics of succinic dehydrogenase, namely that k_3' may play an important rather than an insignificant part in determining the value of K_m. This view was first put forward by Briggs & Haldane (1925) on theoretical grounds, and has since been shown by Slater & Bonner (1952) to apply in the case of this enzyme.

The high relative affinities reported by Potter & DuBois (1943), using rat-liver homogenates as a source of enzyme, and by Krebs *et al.* (1952), using a yeast preparation, are what might be expected for highly active succinic oxidase systems, in the light of the results given here. However, K_m of the enzyme in homogenates is given by Ackermann & Potter (1949) as about 10^{-2}M, and the K_i of malonate about 10^{-4}M. Similarly, data given by Krebs *et al.* (1952) on the effect of succinate concentration on the oxygen consumption of the yeast system yield a value for K_m of about 10^{-2}M. Even if these K_m values were corrected for the effect of competitive inhibition by phosphate (Slater & Bonner, 1952) they would still be of a different order of magnitude from those found in heart-muscle preparations. The explanation of these facts, and of the affinity ratio of 10 found by Krebs & Johnson (1948), using pigeon breast-muscle suspensions, is not apparent from the work reported here, and must await further investigation.

SUMMARY

1. Using potassium ferricyanide as hydrogen acceptor, the Michaelis constant (K_m) of succinic dehydrogenase in six different heart-muscle pre-

parations had values ranging from $2{\cdot}5 \times 10^{-4}$M to $5{\cdot}3 \times 10^{-4}$M.

2. In the same series of experiments, the inhibitory constant (K_i) of malonate had values ranging from $5{\cdot}4 \times 10^{-6}$M to $9{\cdot}8 \times 10^{-6}$M.

3. The ratio of affinities of succinic dehydrogenase for malonate and succinate (K_m/K_i), determined directly, varied from 4·7 to 60, depending on the nature and concentration of the hydrogen acceptor. This variation can be explained by differences in the value of the rate constant (k_3') for the decomposition of the enzyme-substrate complex to give the products of the reaction.

4. The true ratio of affinities of succinic dehydrogenase for malonate and succinate, in the absence of further reaction of succinate, is about 3.

5. Values of the rate constants of the uninhibited reaction have been calculated, and were found to agree substantially with those previously reported.

6. The results have been briefly discussed in relation to estimates of the ratio of affinities (K_m/K_i) by other workers using different types of preparation.

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The Metabolism of Ethers in the Rabbit

1. ANISOLE AND DIPHENYL ETHER

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Investigations in this laboratory of the metabolic fate of aromatic compounds containing 'potential centres for conjugation' (see Bray, Ryman & Thorpe, 1948; Thorpe, 1950) have been concerned mainly with compounds containing groups which may be converted to carboxyl in the animal body, e.g. $-CH_3$; $-CONH_2$; $-CN$. Another common conjugable group, the hydroxyl group, might theoretically be formed *in vivo* from ethers. The evidence in the literature suggests that the ether link in unsubstituted alkyl phenyl ethers is not hydrolysed to any appreciable extent *in vivo*, since Kossel (1880, 1883) and Lehmann (1889) found that a glucuronide of ethyl *p*-hydroxyphenyl ether was a major metabolite of phenetole (ethyl phenyl ether) in the dog. With substituted alkyl phenyl ethers, however, there is evidence that considerable dealkylation may occur *in vivo*, as in the following examples: the formation of *p*-iodophenol from *p*-iodoxyphenyl methyl ether (Röhmann, 1905); of *p*-aminophenol from phenetidine (*p*-aminophenyl ethyl ether) and phenacetin, its *N*-acetyl derivative (Müller, 1888; Mörner, 1889; Smith & Williams, 1949*a*, *b*); of *p*-hydroxydiphenyl from *p*-methoxydiphenyl and of *p*-hydroxyphenyl *p*-methoxyphenyl ether from di-(*p*-methoxyphenyl) ether (Stroud, 1940*a*). Stroud did not observe fission of a phenyl group and there appears to be no example in the literature of such fission of diphenyl ethers. It was thus of interest to investigate the metabolism of aromatic ethers in detail. Anisole (methyl phenyl ether) and diphenyl ether have been chosen

for study as examples of unsubstituted aromatic ethers. The fate of the former does not appear to have been investigated previously, apart from the observation of Coombs & Hele (1926) that it caused an increased excretion of ethereal sulphate and, so far as we are aware, the only information about the fate of diphenyl ether is the observation of Stroud (1940*b*) that, when this ether is injected into rabbits, 23% is excreted in urine as *p*-hydroxyphenyl phenyl ether and that none of the diphenyl ether is excreted unchanged. In the present investigation the effect of anisole and diphenyl ether on the excretion of ethereal sulphate, glucuronic acid, phenols and mercapturic acid has been determined and the major metabolites isolated and characterized.

MATERIALS AND METHODS

Materials. Anisole, diphenyl ether and *o*- and *p*-methoxyphenol were purchased (British Drug Houses Ltd.). The *o*- and *p*-hydroxyphenyl phenyl ethers used (m.p. 106° and 82° respectively) were prepared by the action of potassium phenoxide on *o*- and *p*-bromophenol in the presence of copper bronze at 180–200° (Lock, 1930). Di-(*p*-hydroxyphenyl) ether (m.p. 161°) was prepared from di-(*p*-nitrophenyl) ether (Eastman Kodak Co. Ltd.) via di-(*p*-aminophenyl) ether and the corresponding diazonium salt (Häussermann & Teichmann, 1896; Häussermann & Bauer, 1897). For the characterization of phenols isolated as metabolites the *p*-nitrobenzyl ethers, carbanilates and phenoxyacetates were prepared as described by Johnson, Shennan & Reed (1944). The m.p.'s of these derivatives are given in the appropriate sections below. p-*Methoxyphenyl* p-*nitrobenzyl ether* forms pale yellow leaflets (m.p. 87°). (Found: OMe, 12·1; $C_{14}H_{13}O_4N$ requires OMe, 12·0%.) p-*Methoxyphenyl carbanilate* forms colourless leaflets (m.p. 133°). (Found: OMe, 13·0; $C_{14}H_{13}O_3N$ requires OMe, 12·8%.)

Animals, diet and dosage. The rabbits used were 2–3 kg. does. They were maintained throughout the experimental period on the diet of pellets and water as previously described (Bray, Ryman & Thorpe, 1947). The ethers were administered with water by stomach tube at dose levels of 0·4–0·5 g./kg. No toxic effects were observed.

Analytical methods. Ethereal sulphate was estimated by the gravimetric method of Folin (1905), ether and ester glucuronide by the Shaffer-Hartmann method described previously (Bray, Neale & Thorpe, 1946), but using 10N-H_2SO_4 (0·5 vol.) for the hydrolysis; glucuronic acid by a modified naphthoresorcinol method (Bray, Humphris, Thorpe, White & Wood, 1952); and free and total phenols by a modification of the Folin & Ciocalteu method (Bray, Thorpe & White, 1950*b*). Mercapturic acid (iodine consumption after hydrolysis by alkali) was estimated by Stekol's method (1936).

Column chromatography. Fractionation of urine extracts was carried out using a column (2·5 cm. diameter, 60 cm. long) packed with hydrocellulose (Imperial Chemical Industries Ltd.), or powdered Whatman filter tablets, in conjunction with a mechanical fraction collector.

Paper chromatography. The methods were as described by Bray, Thorpe & White (1950*a*). Whatman no. 4 paper was used. The solvent mixtures were: *A*, light petroleum (b.p. 40–60°) saturated with formic acid; *B*, light petroleum (b.p. 90°) 50%, formic acid 20%, water 30% (all v/v); *C*, light petroleum (b.p. 90°) 48%, *n*-butanol 4%, formic acid 48%; *D*, light petroleum (b.p. 90°) saturated with ammonia (sp.gr. 0·880); *E*, benzene 50%, acetic acid 33%, water 17%. Detection of phenols was most satisfactory with diazotized *p*-nitraniline and K_2CO_3 (20% w/v) which gave spots with *o*-, *m*- and *p*-methoxyphenol which were purple, red and grey-blue with pink edges respectively. In runs of 16 hr. *p*-methoxyphenol gave R_F 0·33, 0·24 and 0·30 in solvent mixtures *A*, *B* and *C* respectively. (Corresponding R_F for *m*-methoxyphenol 0·4, 0·37 and 0·50.) No satisfactory solvent mixture was found for guaiacol or its azo dye formed by coupling with diazotized *p*-nitraniline. Both *o*- and *p*-hydroxyphenyl phenyl ethers moved with the solvent front in all solvent mixtures tried. The purple azo dye from *p*-hydroxyphenyl phenyl ether gave R_F 0·6 when run 1 hr. in solvent mixture *D*, but the dye formed from the *o*-isomer did not give satisfactory chromatograms. Di-(*p*-hydroxyphenyl) ether gave R_F 0·6 (red spot) when run 0·75 hr. in solvent mixture *E*, and its azo dye R_F 0·01 when run 3 hr. in solvent mixture *D*.

RESULTS

Quantitative experiments

Normal excretion of metabolites. The average daily excretion of ethereal sulphate by individual rabbits ranged from 28 to 55 mg. SO_3 and the average percentage by which the individual daily normal values differed from the weekly average used for base-line for calculation of final results was ±9% (3·5 mg. SO_3). The corresponding values for reducing material after hydrolysis (expressed as glucuronic acid) were 272–486 mg. and ±18%, for glucuronic acid by the naphthoresorcinol method 130–301 mg. and ±8%. For phenols (expressed as and using as standard *p*-methoxyphenol) the values were free 135–173 mg. and ±15%, conjugated 31–83 mg. and ±14%; for phenols (expressed as and using as standard *p*-hydroxyphenyl phenyl ether) free 224–279 mg. and ±7%, conjugated 116–196 mg. and ±24%.

Metabolites of anisole and diphenyl ether. The quantitative results obtained after administration of the two ethers are summarized in Table 1. It can be seen that the only metabolites indicated by the analytical results are phenols, excreted either free or as *O*-conjugates and accounting together for approximately 80% of the dose. The fate of the remainder is unknown. Unchanged ethers were not detected in the urines and no smell of anisole or diphenyl ether was detected in the breath of the animals. There was no significant iodine consumption by alkali-hydrolysed urine, i.e. probably no mercapturic acid formation.

Qualitative experiments

Anisole. Continuous extraction with ether of urine as collected from rabbits which had each received anisole (1·5 ml.) gave an extract which,

after evaporation of the solvent, left a syrupy residue in which *p*-methoxyphenol was shown, by paper chromatography, to be the main phenol. Comparison with paper chromatograms of standard *p*-methoxyphenol solutions suggested that not more than 8 % of the dose was present in this form. The 24 hr. urine of nine rabbits similarly dosed was hydrolysed by boiling under reflux for 1 hr. with hydrochloric acid (0·2 vol. 10N). After cooling it was continuously extracted with ether for 72 hr.

Table 1. *Amounts of metabolites excreted in urine in 24 hr. by rabbits after administration of anisole and of diphenyl ether*

(Dose 0·5 g./kg. Results are expressed as percentage of the dose, ranges are given in parentheses and the number of experiments indicated by superior figures.)

Type of metabolite	Anisole	Diphenyl ether
Ethereal sulphate	29 (24–31)^3	12 (11–13)^3
Ether glucuronide	55 (31–65)^6	69 (58–76)^6
Glucuronic acid	41 (35–47)^6	57 (53–61)^2
Free phenols	4 (1–7)^4	15 (10–18)^4
Conjugated phenols	78 (76–80)^4	64 (55–71)^4
Total phenols by phenol estimation	82	79
Total phenols by estimation of conjugating acids and free phenols	81	90

The brown syrup left after removal of solvent from the extract was dissolved in acetone and chromatographed, using a paper powder column with benzene as eluent. The 38 hourly portions (approx. 50 ml. each) collected were grouped into fractions in which the phenolic constituents were identified by paper chromatography. These fractions, with the hours of collection and the phenols identified, were: *A*, 1–5 hr. no phenols; *B*, 6–10 hr., *C*, 11–15 hr., *D*, 16–19 hr. and *E*, 20–24 hr. *p*-methoxyphenol; *F*, 25–27 hr. *o*- and *p*-methoxyphenol; *G*, 28–38 hr. phenols of normal urine including quinol. The amount of quinol found in fraction *G* corresponded with that found in extracts of hydrolysed normal rabbit urine to which *p*-methoxyphenol had been added, so that the quinol in this fraction can be regarded as that normally excreted by rabbits and not as derived from *p*-methoxyphenol. The phenolic fractions were concentrated under reduced pressure to give syrups, some of which crystallized on standing. Derivatives of methoxyphenol were obtained from these; for example, *B* (0·5 g.) yielded *p*-methoxyphenyl carbanilate, m.p. 133° (0·34 g.); *C* (3·7 g.) *p*-methoxyphenyl-*p*-nitrobenzyl ether, m.p. 86° (2·9 g.); *E* (0·5 g.) *p*-methoxyphenoxyacetic acid, m.p. 106° (0·03 g.). From fraction *F* (0·5 g.), which also contained *p*-methoxyphenol, a small amount (14 mg.) of *o*-methoxyphenoxyacetic acid, m.p. 117°, was separated. None of these derivatives depressed the melting point of authentic specimens.

Attempted isolation of a glucuronide. Treatment of the urine by the usual lead precipitation procedure for the isolation of a glucuronide (cf. Bray *et al.* 1947) gave only a syrup which could not be induced to crystallize. It gave a strongly positive naphthoresorcinol test. An aqueous solution of the syrup, after extraction with benzene to remove unconjugated phenols, was hydrolysed by boiling under reflux with sulphuric acid (equal vol. 10N) for 1·5 hr. and then extracted with ether. The extract was fractionated on a hydrocellulose column, eluting with benzene. *p*-Methoxyphenol was isolated as its *p*-nitrobenzyl ether. A small amount (6 mg. from 8 g. anisole administered) of *o*-methoxyphenyl *p*-nitrobenzyl ether, m.p. 61°, was also obtained.

Phenol as a metabolite of anisole. It was important to decide whether phenol was a metabolite of anisole in the rabbit. No satisfactory solvent mixture has been found for the identification of phenol by paper chromatography, but mixtures of phenol and *p*-methoxyphenol can be clearly separated on a hydrocellulose column using as solvent a mixture of light petroleum (b.p. 90°) (10 vol.), *n*-butanol (1 vol.) and 98 % formic acid, 2 % water (5 vol.). From ether extracts of urine hydrolysed by boiling for 1 hr. under reflux with sulphuric acid (equal vol. 10N) separate fractions were collected every 10 min. No unsubstituted phenol was detected in any of the fractions either from normal rabbit urine or from urine collected after administration of anisole. If, however, anisole containing small amounts of phenol was given (purchased specimens of anisole may contain more than 2 %), some fractions giving a reaction for unsubstituted phenol were obtained. Quantitative estimation of phenol in the combined fractions from such an experiment indicated an amount corresponding to 70 % of that administered which was 27 mg./g. anisole.

Diphenyl ether. Paper-chromatographic examination of ether extracts of acid-hydrolysed urines from rabbits dosed with diphenyl ether indicated that *p*-hydroxyphenyl phenyl ether was the principal metabolite. Continuous ether extraction (72 hr.) of the pooled 24 hr. urines as collected (pH 7·8) from nine rabbits each dosed with diphenyl ether (1 g.) gave an extract from which *p*-hydroxyphenyl phenyl ether (m.p. 80°, 30 mg.) was obtained. (Mixed m.p. with authentic specimen, 80°.) The residual urine was hydrolysed by boiling under reflux 1 hr. with hydrochloric acid (0·2 vol. 10N) and then continuously extracted with ether for 72 hr. An aqueous solution of the ether-soluble material was extracted six times with benzene (5 ml.). The extracts were combined and concen-

trated under reduced pressure to 15 ml. On keeping at 0° platelets separated (compound *A*). Benzene was removed from the mother liquor and the residue recrystallized from water and methanol. *p*-Hydroxyphenyl phenyl ether (m.p. 82°) was obtained. (Yield, 1 g.) No *o*-hydroxyphenyl phenyl ether was detected. Compound *A* after recrystallization from water had m.p. 156°. (Yield, 8 mg.) It reduced ammoniacal silver nitrate and reacted in the same way as di-(*p*-hydroxyphenyl) ether on paper chromatograms. Mixed m.p. with authentic di-(*p*-hydroxyphenyl) ether (m.p. 161°) 156–160°. (Found: C, 70·1; H, 5·0. Calc. for $C_{12}H_{10}O_2$: C, 71·3; H, 5·0%.) Compound *A* was, therefore, probably impure di-(*p*-hydroxyphenyl) ether.

Phenol could not be detected in the urine by the methods described for anisole urine. The amount of quinol detected by paper chromatograms was no greater than that found in normal rabbit urine.

Isolation of a glucuronide. Application of the lead procedure to urine from rabbits dosed with diphenyl ether gave a syrup which partially crystallized and from which after recrystallization from aqueous ethanol (charcoal) colourless needles, m.p. 174°, were obtained. (Yield, 1·03 g., from 10 g. diphenyl ether.) They gave a strongly positive naphthoresorcinol test and were readily soluble in ethanol, $[\alpha]_D^{24°} - 70° \pm 6°$ in water (*c*, 0·172). (Found: C, 57·0; H, 5·3; H_2O, 4·9%; equiv. wt. 371. *p-Phenoxyphenyl glucuronide monohydrate*, $C_{18}H_{18}H_8 \cdot H_2O$ requires C, 56·8; H, 5·3; H_2O, 4·7%; equiv. wt. 380. *p*-Hydroxyphenyl phenyl ether was isolated from the hydrolysed glucuronide.

DISCUSSION

From the foregoing experiments it is clear that, in the two unsubstituted ethers examined, fission of the ether linkage in the rabbit does not occur to a significant extent. (The method used for detection of unsubstituted phenol should have revealed an excretion of 3 mg./rabbit, or fission of 0·3% of the ether administered.) The chief changes which these compounds undergo *in vivo* involve the formation of a new centre for conjugation by hydroxylation, with conjugation of the resultant phenolic ethers with glucuronic and sulphuric acids. Over 80% of the administered compounds can be accounted for in this way. The phenolic ethers formed were predominantly *p*-isomers, small amounts of *o*-isomer being detected only after administration of anisole. These findings are in accord with those of previous workers on phenetole (Kossel, 1880, 1883; Lehmann, 1889) and on diphenyl ether (Stroud, 1940*b*). They do not support Williams's (1947) interpretation of the results of Coombs & Hele (1926) in which he attributes the increased excretion of ethereal sulphate by the dog after ingestion of anisole to conjugation of phenol formed by splitting of the ether linkage. It is clear from our experiments that in the rabbit this ethereal sulphate is almost entirely formed from *p*-hydroxyphenyl methyl ether, and that the *o*-isomer and phenol itself can only contribute to a very small extent. The evidence available suggests that the ether linkage of unsubstituted alkyl phenyl ethers and diphenyl ether is not readily split *in vivo* in the rabbit and the dog.

A study of the fate of several substituted methyl ethers in the rabbit is in progress in this laboratory and indicates that in some such compounds, e.g. methyl *p*-nitrophenyl ether, extensive fission of the ether linkage, comparable in extent to that found by Stroud (1940*a*) for di-(*p*-methoxyphenyl) ether and *p*-methoxydiphenyl, may occur. In such compounds the methoxy group may be regarded as a potential centre for conjugation. These experiments will be presented in a later paper.

SUMMARY

1. The fate of anisole (methyl phenyl ether) and diphenyl ether in the rabbit has been studied.

2. These ethers are hydroxylated and the resultant phenols are excreted unconjugated and conjugated with glucuronic and sulphuric acids to the extent of 2, 48 and 29% of the dose for anisole and 15, 63 and 12% for diphenyl ether respectively.

3. The chief metabolite of anisole is *p*-methoxyphenol but a small amount of *o*-methoxyphenol is also formed. No fission of the ether linkage was detected.

4. The chief metabolite of diphenyl ether is *p*-hydroxyphenyl phenyl ether but a small amount of another metabolite probably di-(*p*-hydroxyphenyl) ether is also formed. No fission of the ether linkage was detected.

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The Hydrolysis of Esters of Phenol by Cholinesterases and Other Esterases

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In recent years it has been shown that cholinesterases of both types can catalyse the hydrolysis of many non-choline aliphatic esters (for literature see Whittaker, 1951). The primary object of the present investigation was to find out if cholinesterases can also hydrolyse non-choline esters possessing an aromatic nucleus.

A series of *p*-substituted phenyl acetates was selected for study. It was found that these esters are indeed hydrolysed rapidly by the purified cholinesterases of human erythrocytes and plasma and by cobra-venom cholinesterase. During the course of the work it became apparent, however, that, as with aliphatic esters, the hydrolysis of the aromatic esters by whole human erythrocytes and plasma could be accounted for only in part by the cholinesterase content of these preparations. Some additional studies were therefore made of the other esterases involved.

The hydrolysis of aromatic esters by blood and tissues has been the subject of several previous studies, but little attempt has been made to characterize the enzymes responsible for hydrolysing them. The hydrolysis of short-chain aliphatic esters of *p*-nitrophenol (Huggins & Lapides, 1947; Huggins & Moulton, 1948) and of 2-naphthol (Nachlass & Seligman, 1949; Seligman, Nachlass & Mollomo, 1949) was attributed by these authors to aliphatic esterases (ali-esterases). Augustinsson (1948, 1950) studied the hydrolysis of acetylsalicylic acid, acetylsalicylylcholine and salicylylcholine by cholinesterase preparations derived from the blood, brain and tissues of various species, but no special precautions were taken to exclude other esterases. Zeller, Fleisher, McNaughton & Schweppe (1949) showed that preparations of snake venom and erythrocyte cholinesterase free from ali-esterase could hydrolyse phenyl acetate and *p*-nitrophenyl acetate, and that the hydrolysis was fully eserine-sensitive, the latter constituting good evidence that the cholinesterase was responsible. Diluted whole human plasma, however, split phenyl acetate very much faster than acetylcholine and the hydrolysis was but little depressed by eserine, a result consistent with that of Huggins & Lapides (1947) with *p*-nitrophenyl acetate. The hydrolysis of *m*-acetoxyphenyltrimethylammonium methosulphate by human serum and erythrocytes and rat brain was reported by Wescoe, Riker & Beach (1950). Again,

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no precautions were taken to exclude non-choline esterases.

Aldridge (1951*a*, *b*, 1953) has studied the hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by the sera and tissues of a wide range of species. In addition to any contribution made by cholinesterase, two other enzymes, named by him the A- and B-esterases, present in varying proportions in different sera, hydrolyse these substrates. The B-esterase, like the cholinesterases, is sensitive to toxic organophosphorus compounds; it hydrolyses butyrates faster than acetates. The A-esterase is not only insensitive to organophosphorus compounds; it has the remarkable property of hydrolysing at least one of them, diethyl *p*-nitrophenyl phosphate (E 600). The relation of these findings to our own work is discussed below.

MATERIALS AND METHODS

Substrates. Acetyl-β-methylcholine (MCh; β-acetoxypropyltrimethylammonium chloride) and acetylcholine (ACh) were obtained commercially; they were used either without further purification or after conversion to the perchlorates (Bell & Carr, 1947). Phenyl acetates were prepared from the corresponding phenol and acetic anhydride in the presence of aqueous NaOH (Chattaway, 1931). *p-tert.*-Butylphenol was prepared as described by Liebmann (1881) and *p*-acetoxyphenyltrimethylammonium iodide (*p*-APTA) by warming *p*-dimethylaminophenyl acetate with methyl iodide.

Inhibitors. Eserine was obtained commercially; di*iso*propylphosphorofluoridate (DFP), $(Pr^{i}O)_2POF$, was kindly provided by Dr D. R. Davies, Porton, and E 600 by Dr W. N. Aldridge, Carshalton. Commercial samples of these inhibitors were also used. The DFP was used without further purification; E 600 was freed from hydrolysis products and tetraethyl pyrophosphate by extracting an ethereal solution with Na_2CO_3, drying and removal of ether *in vacuo*.

*Human-erythrocyte aceto-cholinesterase**. Three preparations of differing degrees of purity were made from erythrocytes from time-expired or Kahn-positive human blood kindly supplied by the Oxford Blood Bank and the Blood Bank of the University of Cincinnati.

(*A*) *Crude preparation.* Laked cells were washed three times with 0·9% (w/v) NaCl and lysed in distilled water. This preparation contained, in addition to the aceto-cholinesterase, an aliphatic esterase (ali-esterase) and (as will be shown below) an aromatic esterase distinct from the other two esterases and capable of hydrolysing the phenyl acetates. Q_{MCh} (μl. acid liberated/mg. dry wt./hr. in presence of 30 mM-acetyl-β-methylcholine), 12.

(*B*) *Partially purified preparation.* Preparation *A* was brought to pH 5·9 and the precipitated stromata collected by centrifuging at 3000 *g*. They were washed with water and 0·1 M-phosphate buffer, pH 6·1, until the supernatant was almost colourless. After a final wash with water they were resuspended in water, adsorbed on to kieselguhr and eluted with 0·2% (w/v) $NaHCO_3$ as previously described (Mounter & Whittaker, 1950). Q_{MCh}, 380; purification, 32-fold; yield, 53%. This preparation hydrolysed tributyrin at less than 1% of the acetyl-β-methylcholine rate and was therefore free from ali-esterase by the criteria adopted in the previous specificity studies of Mounter & Whittaker (1950).

(*C*) *Further-purified preparation.* Preparation *B* was further purified as follows. The cholinesterase was collected by centrifuging at 16000 *g*. for 30 min., dispersed in 0·2% (w/v) $NaHCO_3$ and incubated for 12 hr. at 38° with lysolecithin (King & Dolan, 1933). Inactive material was removed by centrifuging at 16000 *g*. for 30 min. The cholinesterase was recovered from the clear supernatant by precipitation at 50% saturation with $(NH_4)_2SO_4$, resuspended in water, dialysed and made 0·2% with $NaHCO_3$. Q_{MCh}, 1060; purification, 83-fold, yield 18%.

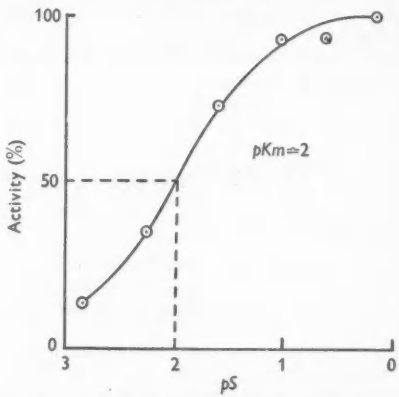

Fig. 1. Hydrolysis of *p*-acetoxyphenyltrimethylammonium iodide by human-erythrocyte aceto-cholinesterase as a function of substrate concentration. Abscissa, negative logarithm of molar substrate concentration (*pS*); ordinate, activity as percentage of that at $pS = 0$.

Cobra-venom aceto-cholinesterase. The cholinesterase of cobra venom has recently been shown to be a typical aceto-cholinesterase (Mounter, 1951), and it is unaccompanied by ali-esterase. The same sample of venom was used as in the earlier experiments without purification.

Butyro-cholinesterase. Human plasma was used as the source of butyro-cholinesterase. Experiments were carried out with (*a*) whole plasma, (*b*) a partially purified preparation free from ali-esterase, obtained by following Strelitz's (1944) procedure to the end of stage 3, (*c*, *d*) Cohn plasma fractions IV-6 and IV-6-3, kindly supplied by Dr D. M. Surgenor, Harvard University. Dilution of the more active preparations was made in 0·2% (w/v) $NaHCO_3$ containing 1% (w/v) gum acacia.

Measurements of esterase activity. These were normally carried out at 37° and pH 7·4 by the Warburg manometric technique as modified by Ammon (1933) with the precautions and controls described in previous publications (Sturge & Whittaker, 1950; Mounter, 1951). In some experiments the colorimetric method of Hestrin (1949) was used to follow the rate of disappearance of substrate. 100 μmoles of the phenyl esters or 30 μmoles of the choline

* For definition of 'aceto-cholinesterase' see Sturge & Whittaker (1950).

esters were used/ml. reaction mixture, unless otherwise stated. In the cobra-venom experiments the esters were first emulsified in 5% (w/v) gum acacia before use (Mounter, 1951).

In inhibitor experiments, the inhibitor was allowed to stand in contact with the enzyme for 20 min. at room temperature and 20 min. at 37°, before addition of substrate. The substrate concentrations used were sufficient to ensure maximum rates of hydrolysis in each case. While complete velocity-substrate concentration studies have not been carried out for all substrates, the results with *p*-APTA (Fig. 1), previous results with the choline esters and a few additional observations with other substrates justify this statement.

RESULTS

Erythrocytes

The ability of human-erythrocyte cholinesterase to hydrolyse phenyl acetates was first studied with preparation *B*, which was free from ali-esterase (Table 1). It will be seen that phenyl acetate is split

Table 1. *Hydrolysis of* p-*substituted phenyl acetates by a preparation of erythrocyte cholinesterase free from ali-esterase* (*preparation B*)

Substituent	Rate of hydrolysis (% MCh rate)	Inhibition by 10 μM-eserine (%)	Inhibition by 1 μM-DFP (%)
H	300	—	61
Me	253	60	—

three times as fast as acetyl-β-methylcholine or about as fast as acetylcholine at optimum substrate concentration. Competition experiments (Table 3) showed that a mixture of phenyl acetate and acetyl-β-methylcholine was hydrolysed at a rate intermediate between those obtained with each substrate separately, thus suggesting that both substrates were competing for the same active centres. When inhibitor experiments were carried out, however, it was found that the hydrolysis of the phenyl acetates was not completely inhibited by concentrations of DFP and eserine which completely inhibited the acetyl-β-methylcholine hydrolysis (Table 1). It was suspected, therefore, that part of the hydrolysis of the phenyl acetate by preparation *B* was due to an esterase which is distinct from both the cholinesterase and the aliphatic esterase and which had not been removed along with the latter during the purification procedure. We propose to refer to this esterase as the aromatic esterase.

Table 3. *Competition of phenyl acetate* (PhAc) *and acetyl-β-methylcholine for erythrocyte cholinesterase*

Enzyme source	Rate (μl./30 min.) in presence of MCh	PhAc	MCh + PhAc
A. Laked erythrocytes	495	1690	1580
B. Partially purified	680	2160	1320
C. Further purified	970	1860	1180

Studies were then continued with the more highly purified cholinesterase preparation (*C*). Table 2 shows the rate of hydrolysis of a number of *p*-substituted phenyl esters by this preparation. It will be seen that the rate of hydrolysis of phenyl acetate relative to acetyl-β-methylcholine has dropped from 300 to 192%, and the hydrolysis is completely or almost completely inhibited by concentrations of the three anti-cholinesterases, DFP, eserine and E 600 which are known to produce complete inhibition of cholinesterase activity. We believe, therefore, that the results in col. 2, Table 2, represent the rate at which these aromatic esters are hydrolysed by the red-cell cholinesterase relative to acetyl-β-methylcholine. It will be seen that all the esters are hydrolysed at appreciable rates, four

Table 2. *Hydrolysis of* p-*substituted phenyl acetates by different enzyme preparations*

(Rate of hydrolysis expressed as percentage of rate with acetyl-β-methylcholine (erythrocyte and cobra-venom preparations) or acetylcholine (plasma). Inhibitors used at the following concentrations: eserine, 10 μM, DFP, 1 μM, E 600, 1 μM.)

Substituent	Erythrocyte cholinesterase (preparation *C*) Rate of hydrolysis	Inhibition by Eserine (%)	DFP (%)	E 600 (%)	Cobra venom Rate of hydrolysis	Inhibition by eserine (%)	Whole human plasma Rate of hydrolysis	Inhibition by eserine (%)	Human plasma butyrocholinesterase (preparation *b*) Rate of hydrolysis
H	192	96	100	99	138	97	720	12	91
Cl	63	—	100	—	26	—	—	—	28
Br	53	—	—	—	—	—	—	—	28
I	133	—	—	—	63	97	—	—	28
Me	157	98	98	—	36	100	450	14	65
MeO	218	—	—	99	126	100	—	—	51
tert.-Bu	69	—	97	—	—	—	—	—	15
Me_3N^+	49	—	98	—	—	—	—	—	95
NO_2	—	—	—	—	—	—	—	—	36

faster than acetyl-β-methylcholine. *p*-Methoxyphenyl acetate is the most rapidly hydrolysed; phenyl acetate follows, and *p*-iodophenyl and *p*-cresyl acetate are also split more rapidly than acetyl-β-methylcholine.

The aromatic esterase of erythrocytes. Some properties of the aromatic esterase were studied using unpurified laked erythrocytes (preparation *A*). The earlier results (Table 1) had indicated that this enzyme was relatively insensitive to DFP. This was confirmed in the experiments presented in Fig. 2. Increasing DFP concentrations caused a steep initial fall in the rate of phenyl acetate hydrolysis which was complete at 1 μM, a concentration which, as shown by the MCh data inserted for comparison, completely inhibits cholinesterase activity. Thereafter, little further fall in the rate of phenyl acetate hydrolysis occurred even when the DFP concentration was increased 100-fold. This residual DFP-

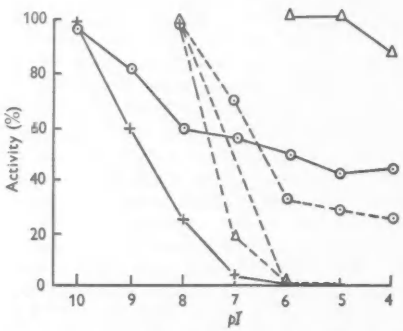

Fig. 2. Effect of eserine (continuous lines) and DFP (broken lines) on hydrolysis of phenyl acetate (⊙), acetyl-β-methylcholine (+) and tributyrin (△) by laked human erythrocytes. Ordinates, activity expressed as a percentage of uninhibited activity; abscissa, negative logarithm of molar inhibitor concentration (*pI*).

insensitive activity must be attributed to a DFP-insensitive aromatic esterase. The fact that tributyrin hydrolysis was almost completely inhibited by 1 μM-DFP again shows that the aromatic esterase is distinct from the aliphatic esterase hydrolysing tributyrin.

In similar experiments with eserine, the cholinesterase is completely inhibited at 1 μM-inhibitor concentration while the phenyl acetate hydrolysis is only partially inhibited at many times this concentration. It should be noted, however, that the aliphatic esterase is also unaffected by this inhibitor, and that the portion of the phenyl acetate hydrolysis insensitive to 1 μM-eserine is greater than the portion insensitive to 1 μM-DFP. This suggests that a small portion of the phenyl acetate hydrolysis is due to the aliphatic esterase. Taking the activity abolished by 1 μM-eserine as that due to the cholinesterase and the activity abolished by 1 μM-DFP as that due to cholinesterase plus ali-esterase, the contribution made by the three esterases to the hydrolysis of each of the three substrates may be calculated. The results are presented in the form of block diagrams in Fig. 3. The portion of the total phenyl acetate hydrolysis attributed to cholinesterase in this figure, when expressed as a percentage of the acetyl-β-methylcholine hydrolysis, gives a value identical with that obtained with purified cholinesterase (Table 2).

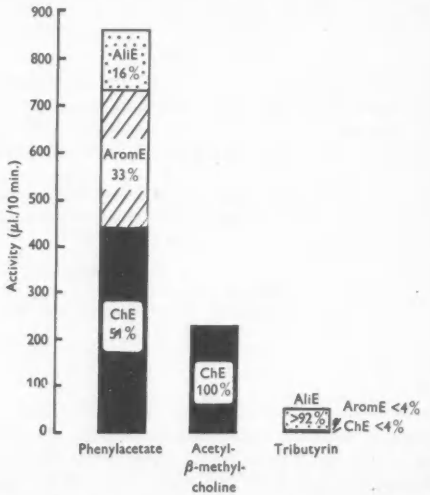

Fig. 3. Contribution made by cholinesterase (ChE), aromatic esterase (AromE) and aliphatic esterase (AliE) of laked human erythrocytes to hydrolysis of phenyl acetate, acetyl-β-methylcholine and tributyrin. The percentage contribution made by each enzyme to the total hydrolysis of each substrate is indicated within the histograms.

It is interesting to note that about 16% of the total phenyl acetate hydrolysis is attributed to the aliphatic esterase; since the total rate of hydrolysis of phenyl acetate is so much higher than that of tributyrin, phenyl acetate is actually split nearly three times as fast as tributyrin by this esterase. By contrast, the results with tributyrin show that this substrate is attacked at less than 1% of the rate of phenyl acetate by the aromatic esterase. *iso*Amyl acetate and *n*-hexyl acetate are also split by it at a negligible rate (Table 5).

The specificity of the aliphatic and aromatic esterases towards other phenyl acetates was next studied; the results are presented in Table 4, and were obtained as follows. The aromatic esterase figures were obtained as before by measuring the

rate of hydrolysis of the esters by laked erythrocytes in the presence of 1 μM-DFP. The figures for the aliphatic esterase were obtained by subtracting from the DFP-sensitive portion of the total hydrolysis the contribution made by the cholinesterase. This was calculated from the specificity results obtained with preparation *C* (Table 2) and the known cholinesterase activity of the preparation. The results for tributyrin are inserted for comparison.

Table 4. *Specificity of the aromatic and aliphatic esterases of human erythrocytes*

Ester *p*-X.C_6H_4OAc	Rate of hydrolysis of ester (as % of phenyl acetate hydrolysis) by: Aromatic esterase	Aliphatic esterase
X = Cl	149	7
Br	134	2
I	175	18
Me	90	4
MeO	111	5
tert.-Bu	32	7
Me_3N^+	130	0
Tributyrin	<1	35

Table 5. *Hydrolysis of aliphatic and aromatic esters by human erythrocyte-cholinesterase preparations*

Substrate	Rate (% MCh): Laked erythrocytes (*A*)	Partially purified preparation (*B*)	Further-purified preparation (*C*)
Tributyrin	22	<1	<1
n-Hexyl acetate	—	22	21
*iso*Amyl acetate	—	—	70
Phenyl acetate	372	300	192
p-Cresyl acetate	270	253	157

Although some error may attach to results obtained in this indirect way, they serve to show that the aromatic esterase is able to split *p*-substituted phenyl acetates readily, often at a rate surpassing that of phenyl acetate itself. By contrast, *p*-substitution appears to exert an unfavourable effect on hydrolysis by the aliphatic esterase, though the rates of hydrolysis of some of the aromatic esters are by no means negligible when compared with that of tributyrin.

A comparison of the relative rates of hydrolysis of tributyrin and phenyl acetate by the three erythrocyte preparations reveals, in the light of Fig. 3, that the preparation of *B* from *A* and of *C* from *B* correspond to the stepwise removal of the aliphatic and aromatic esterases, respectively. It will be seen in Table 5 that removal of ali-esterase activity from *A* resulted in a fall in the phenyl acetate hydrolysis from 372 to 300 % and in the *p*-cresyl acetate hydrolysis from 270 to 253 %. A preparation containing all of the cholinesterase of *A* and 85–86 % of the aromatic esterase would have just this activity.

The presence of a second carboxylic esterase in an erythrocyte cholinesterase preparation, judged enzymically homogeneous by previous criteria, raises the question as to whether the earlier specificity results of Adams & Whittaker (1948), Adams (1949) and Mounter & Whittaker (1950) can be considered reliable. The relative rates of hydrolysis of *iso*amyl acetate and *n*-hexyl acetate by the aromatic esterase-free preparation *C* was therefore determined; the results (Table 5) were identical with those obtained earlier.

The equivocal nature of the results given by the competition experiments should be noted. As seen in Table 3, the rate of hydrolysis of a mixture of phenyl acetate and acetyl-β-methylcholine by unpurified laked erythrocyte lies between the rates of hydrolysis of phenyl acetate and acetyl-β-methylcholine separately. This type of result in a competition experiment has often been taken as evidence that both substrates are competing for the same enzyme (cf. Augustinsson, 1950); it clearly does not exclude the possibility that one or both of the pair of competing substrates are also being split by enzymes which do not attack the other substrate. Quantitatively, the results of Table 3 reflect the stepwise elimination of the two non-choline esterases from the laked erythrocytes, but qualitatively, the same type of result was obtained with all three preparations.

Cobra venom

The relevant results presented in Table 2 show no evidence for the presence of an eserine-insensitive aromatic esterase in the crude venom. The absence of ali-esterase has already been noted (Mounter, 1951). All phenyl esters tested were hydrolysed at an appreciable rate and the hydrolysis was inhibited 97–100 % by 10 μM-eserine, a concentration which inhibits the cholinesterase by the same amount (Mounter, 1951). The rates, relative to acetyl-β-methylcholine, were 28–77 % lower than those obtained with the erythrocyte cholinesterase and there were two differences in the order in which the substrates were hydrolysed. The lower rate of hydrolysis may be related to the sharper aliphatic specificity already noted for this enzyme (Mounter, 1951).

Plasma

Whole human plasma hydrolysed phenyl acetates at a high rate relative to acetylcholine but this hydrolysis was inhibited only a few per cent (Table 2; Fig. 4) by 1 μM-DFP and 10 μM-eserine (cf. Huggins & Lapides' (1947) results with *p*-nitrophenyl esters).

Table 6. *Relative rates of hydrolysis of* p*-substituted phenyl acetates by human-plasma butyro-cholinesterase preparations of increasing purity*

Preparation	Q_{ACh}	Rate of hydrolysis (% ACh) of: Phenyl acetate	*tert.*-Butylphenyl acetate	*p*-APTA
(*b*) Oxford preparation	150	91	15	95
(*c*) Harvard plasma fraction IV-6	975	92	12·5	—
(*d*) Harvard plasma fraction IV-6-3	140 000	90	10·5	89*

* Perchlorate.

Partially purified butyro-cholinesterase (preparation *b*) also hydrolysed these esters (Table 2) but at much lower rates relative to acetylcholine and the hydrolysis was now fully sensitive to 10 μM-eserine. Two other human-plasma butyro-cholinesterase preparations (*c* and *d*) of high purity hydrolysed the esters at essentially the same relative rates (Table 6) as the less pure preparation (*b*). It is thus clear that a typical butyro-cholinesterase can hydrolyse these aromatic esters though it accounts for only a few per cent of the hydrolysis of the esters by whole plasma. If the results of Table 2, col. 2, are expressed relative to the maximum rate of hydrolysis of acetylcholine by erythrocytes (ACh/MCh = 3) the relative rates of hydrolysis of the aromatic esters by the two cholinesterases are found to be of the same order of magnitude (16–91 %).

The aromatic esterase of plasma. It has been known for some time that human plasma contains a carboxylic esterase (or esterases) in addition to the butyro-cholinesterase. Adams & Whittaker (1949*a*) reported the presence of an esterase insensitive to 0·1 μM-DFP which accounted for about 10 % of the tributyrin hydrolysis and 20 % of the triacetin hydrolysis of their plasmata. Myers & Mendel (1949) showed that human plasma contains an eserine-insensitive esterase which hydrolyses ethyl chloroacetate. The non-choline esterase has however been regarded as a minor constituent of human plasma in contrast to horse plasma which has a high 'ali-esterase' activity (for literature see Sturge & Whittaker, 1950). The high rate of hydrolysis of certain aromatic esters, unaccounted for by the cholinesterase content of the plasma, suggests that a second esterase distinct from the cholinesterase may, after all, be a major constituent of human plasma. It also raises the question as to the relation of the various non-choline esterase activities of plasma to the esterases of erythrocytes and to each other.

In an attempt to answer some of these points, we have studied the effect of DFP and eserine on the hydrolysis of phenyl acetate in the same way as with laked erythrocytes. As will be seen in Fig. 4, phenyl acetate hydrolysis is depressed to a small and approximately equal extent by concentrations of DFP and eserine which are fully inhibitory to acetylcholine hydrolysis. The DFP- and eserine-sensitive portions of the hydrolysis are in good agreement with the value calculated from the cholinesterase content of the plasma and the phenyl acetate/acetylcholine activity ratio of purified plasma cholinesterase. The esterase mainly responsible for the hydrolysis of phenyl acetate in whole plasma is thus a DFP- and eserine-insensitive enzyme similar to the aromatic esterase of erythrocytes. Tributyrin hydrolysis, by contrast, was fully accounted for by the cholinesterase content of

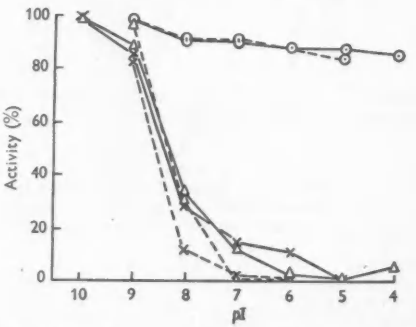

Fig. 4. Effect of eserine (continuous lines) and DFP (broken lines) on hydrolysis of phenyl acetate (⊙), acetylcholine (×) and tributyrin (△) by human plasma. Units as in Fig. 2.

the plasma: its inhibition closely paralleled that of acetylcholine, being negligible at an inhibitor concentration of 1 μM or more, and the tributyrin/acetylcholine activity ratio (46 %) was equal to that of purified butyro-cholinesterase (Adams & Whittaker, 1949*b*). This shows that the aromatic esterase of plasma, like that of erythrocytes, has little or no capacity to hydrolyse tributyrin, and that the eserine-insensitive DFP-sensitive aliphatic esterase of erythrocytes was not present. It is not inconceivable, however, that the aromatic esterase has some action on aliphatic esters, so that the DFP-insensitive tributyrin and triacetin hydrolysis noted by Adams & Whittaker (1949*a*) may have been due to an unusually high aromatic esterase activity in their plasmata.

Relation of the esterases of human blood to the A- and B-esterases

The work so far described has revealed the existence in human blood of two esterases, in addition to the cholinesterases, which participate in the hydrolysis of aromatic esters. One, present in both cells and plasma, is uninhibited by high concentrations of DFP and has little or no action on the typical aliphatic ester, tributyrin. The other, present only in erythrocytes, is sensitive to DFP and

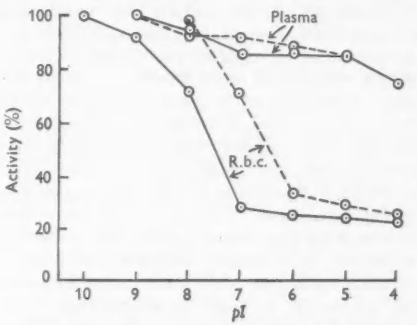

Fig. 5. Effect of E600 (continuous lines) and DFP (broken lines) on hydrolysis of phenyl acetate by human erythrocytes and plasma. Units as in Fig. 2.

hydrolyses tributyrin faster than most aromatic esters tested. Both esterases are relatively insensitive to eserine. Our aromatic esterase somewhat resembles the A-esterase recently described by Aldridge (1951*a*, *b*, 1953) both in hydrolysing aromatic esters and in being insensitive to DFP; the aliphatic esterase may be related to the B-esterase which is sensitive to toxic organo-phosphorus compounds. Little is known of the ability of the A and B enzymes to hydrolyse aliphatic substrates, but like our esterases they are eserine-insensitive. A further point of significance is that the A enzyme alone occurs in human plasma (Aldridge, 1953).

In order to explore these resemblances further, three salient properties of the A- and B-esterases were selected for comparison; their differential sensitivity to E600, their differing acyl group optima and the pH of optimum activity.

E600 *sensitivity.* Fig. 5 shows that the effect of E600 on the hydrolysis of phenyl acetate by erythrocytes and plasma closely parallels that of DFP. The hydrolysis consists of E600-sensitive and E600-insensitive portions approximately equal in magnitude to the DFP-sensitive and -insensitive activities. It follows that the esterases present in these preparations are similarly affected by DFP and E600, i.e. the aromatic esterase, like the A-esterase, is insensitive to E600 while the aliphatic esterase, like the B-esterase, is E600-sensitive.

Acetate/butyrate activity ratios. Another distinguishing feature of the A- and B-esterases is the different relative rates at which they split *p*-nitrophenyl acetate and butyrate. The rate of hydrolysis of phenyl butyrate by the three esterases was measured as before using 1 μM-DFP to inhibit aliesterase and cholinesterase and 1 μM-eserine to inhibit cholinesterase. With the erythrocyte preparation, triacetin was also used as a substrate. The acetate/butyrate ratios were then evaluated and are presented in Table 7. It will be seen that the aromatic esterases of erythrocytes and plasma hydrolysed phenyl acetate much faster than phenyl butyrate; the acetate/butyrate ratio was in fact higher than that found by Aldridge (1951*b*) for the A-esterase of human plasma and esters of *p*-nitrophenol.

Triacetin was hydrolysed at about 15% of the phenyl acetate rate by erythrocyte aromatic esterase. Since tributyrin is hydrolysed at less than 1% of the phenyl acetate rate, the acetate/butyrate ratio for triglycerides must be greater than 15. This suggests that the aromatic esterase may after all have appreciable activity on some aliphatic esters. There is evidence that the A-esterase can slowly hydrolyse ethyl esters (Aldridge, 1951*b*).

The acetate/butyrate activity ratio for the aliphatic esterase of erythrocytes varied, being above unity for the phenyl esters and just less than unity for glyceryl esters. It was, however, much lower than the ratios found with the aromatic esterase. The B-esterases invariably had acetate/butyrate ratios well below unity for *p*-nitrophenyl esters (Aldridge, 1951*b*).

Optimum pH. The optimum pH of the A-esterase is 7·5, with E600 as substrate (Aldridge, 1951*b*).

Table 7. *Acetate/butyrate activity ratios for non-choline esterases of human blood*

	Acetate/butyrate activity ratio			
	Erythrocyte		Plasma	
Esters	Aliphatic esterase	Aromatic esterase	Aromatic esterase	A-esterase*
Phenyl	4·4	22	30	—
Glyceryl	0·98	>15	—	—
p-Nitrophenyl	—	—	—	8

* Aldridge (1951*b*).

The optimum pH of the aromatic esterase was determined using plasma incubated with 1 μM-eserine as the source of the enzyme and phenyl acetate as substrate. The reaction was carried out at different pH values in the presence of 0·05 M-phosphate, and was followed by Hestrin's (1949) colorimetric method for carboxylic esters. As seen in Fig. 6, the optimum pH lies close to 7·5.

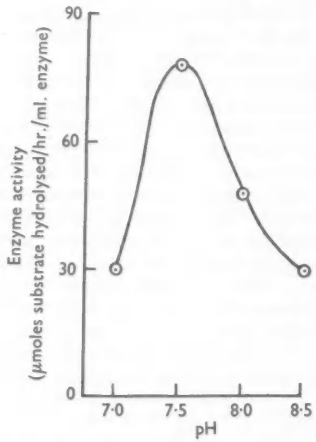

Fig. 6. Effect of pH on hydrolysis rate of phenyl acetate by the aromatic esterase of human plasma.

Three lines of evidence, insensitivity to E 600 and DFP, the acetate/butyrate ratio and the optimum pH thus suggest that our aromatic esterase and Aldridge's A-esterase are identical.

DISCUSSION

The ability of two aceto-cholinesterases and one butyro-cholinesterase to hydrolyse aromatic esters, as exemplified by a series of *p*-substituted phenyl acetates, has been demonstrated. The high rate of hydrolysis of these esters appears to be an exception to the general rule that the rate of hydrolysis of non-choline esters is determined by their degree of structural resemblance to the optimum choline ester. Exceptions to this rule have already been noted and discussed (Mounter & Whittaker, 1950; Whittaker, 1951). The benzene ring is a highly polarizable structure and may be expected to confer an affinity upon these esters greater than that of structural analogues in the aliphatic series. Further, the non-enzymic rate of hydrolysis of all these compounds is higher than for simple aliphatic esters and this may be related to a greater instability of the enzyme-substrate complex.

Two other esterases have been found in human blood, but not in cobra venom, which also hydrolyse phenyl acetate. Both of these are insensitive to eserine but differ in their sensitivity to toxic organophosphorus compounds. One of them appears to be identical with the well known ali-esterase of human erythrocytes and hydrolyses all the esters except phenyl acetate less rapidly than tributyrin. This enzyme is absent from the plasma. The other esterase, here referred to as the aromatic esterase, is present in both erythrocytes and plasma and is characterized both by the low rate at which it attacks glyceryl esters and its high resistance to DFP and E 600. These properties, and the fact that it remained in an erythrocyte cholinesterase preparation which had been freed from ali-esterase, clearly distinguished it from the later enzyme. Proof of the identity of the plasma and erythrocyte aromatic esterases must await their isolation, but if not identical they are at any rate closely similar. Several lines of evidence suggest that the aromatic esterase is probably identical with the E 600-insensitive esterase recently described by Aldridge and named by him the A-esterase. In spite of its low rate of hydrolysis of aliphatic esters, its presence could probably account for all of the so-called aliphatic esterase activity of plasma. The ali-esterase is less certainly but quite possibly to be identified with Aldridge's B-esterase. If this is true our concept of ali-esterase activity will have to be revised. The so-called ali-esterases may emerge as a group of relatively non-specific enzymes which are not limited to attacking aliphatic esters, or even, in the case of at least one member of the group, to carboxylic esters.

The physiological role of these esterases remains obscure. It is possible, however, that they play a part in destroying hormones or drugs whose activity depends on an ester linkage and this possibility may prove relevant to our understanding of the mode of action of such substances.

SUMMARY

1. The ability of two aceto-cholinesterases and one butyro-cholinesterase to catalyse the hydrolysis of a series of *p*-substituted phenyl acetates has been demonstrated.

2. These esters are also attacked by an aromatic esterase present in human erythrocytes and plasma but not in cobra venom.

3. This enzyme attacks aliphatic esters slowly and is not affected by relatively high concentrations of di*iso*propyl phosphorofluoridate or diethyl *p*-nitrophenyl phosphate. It is probably identical with the recently described A-esterase.

4. The well-known ali-esterase of human erythrocytes also appears to hydrolyse the aromatic esters, but in general less rapidly than tributyrin.

It can be separated from the aromatic esterase and is further distinguished from it by its sensitivity to toxic organophosphorus compounds.

5. Phenyl esters are thus hydrolysed by at least five esterases in human blood: erythrocyte acetocholinesterase, plasma butyro-cholinesterase, erythrocyte ali-esterase, erythrocyte aromatic esterase and plasma aromatic esterase. The last two enzymes are either identical or closely similar.

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The Lipids of Fish

3. THE ACETONE-INSOLUBLE FRACTION OF AN ACETONE EXTRACT OF THE FLESH OF THE HADDOCK

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The first paper in this series (Lovern, 1953) described the preparation of successive lipid extracts from the flesh of the haddock, using solvents of increasing effectiveness, and the second paper (Lovern & Olley, 1953) dealt with the acetone-soluble lipids present in the total material extracted by acetone. It was shown that this crude extract also contained much lipid which, after partial purification by reprecipitation procedures, was practically insoluble in acetone at 0°. The present paper describes the examination of this subfraction of the total acetone extract, designated fraction *C* in the previous paper.

ANALYTICAL METHODS

In general the analytical methods used were those described in the previous paper (Lovern & Olley, 1953), but some discussion seems desirable here of N, P and glycerophosphate determination. Mention should also be made of the difficulty of accurate colorimetric determination of low concentrations of plasmalogens (Klenk & Friedrichs, 1952). We have found it preferable, in suitable cases, to determine plasmalogens by direct isolation and weighing of the plasmals, by the procedure of Feulgen, Imhäuser & Behrens (1929), having first removed other unsaponifiable matter by ether extraction of an alkaline hydrolysate of the lipid fraction. We have confirmed that such plasmal extracts give the expected intense aldehyde reaction with the fuchsin reagent, and agreement between the direct colorimetric estimation and the gravimetric estimation is satisfactory.

Nitrogen

Reference was made in the previous paper to the apparent loss of both N and P during hydrolysis of phospholipins. This phenomenon was again encountered with all the fractions described in the present paper. So far as N is concerned, the Kjeldahl digestion mixture (Olley, 1953) was selected because it was found to give quantitative ammonia

production from choline, ethanolamine and serine. It is possible that part of the loss of N after hydrolysis may be due to the formation of unidentified N-containing compounds which are particularly resistant to Kjeldahl digestion. As an example of the extent of this apparent loss, the fraction described as 'unidentified lipid 2' (see below) may be given. This contained 1·6% N by direct Kjeldahl estimation. After hydrolysis by 2 hr. refluxing with 0·5N-ethanolic KOH, and removal of unsaponifiable matter and fatty acids, the N content of the aqueous phase amounted to only 0·8% of the original lipid. When the aqueous phase, which contained some ethanol and ether, and also some excess HCl, was evaporated to dryness under vacuum and the residue taken up in water for subsequent estimation of choline, the total N content had fallen to 0·75%. The unsaponifiable matter, the fatty acids and some insoluble debris separated from the hydrolysate all contained some N. Possible direct loss of N as NH_3 during alkaline hydrolysis was not measured, but after acid hydrolysis of this particular fraction, addition of alkali to the hydrolysate resulted in the evolution of volatile N base (presumably NH_3) equal to 5% of the total original N. Taking into account all the N associated with these various hydrolytic products, and assuming that direct loss as NH_3 during alkaline hydrolysis is of the same order as the evolution of NH_3 on adding alkali to acid hydrolysates, 32% of the total N of 'unidentified lipid 2' remained unaccounted for after alkaline hydrolysis, not including the further loss on vacuum drying.

Phosphorus and glycerophosphate

This same lipid fraction was studied in detail for loss of P during alkaline hydrolysis. The vacuum-dried residue when taken up in water, as described above, has been used in our previous work for the estimation of glycerophosphate. There is usually a little water-insoluble residue produced during vacuum drying of the clear aqueous hydrolysis solution, which was formerly centrifuged off and discarded. It has now been noted that in some samples this insoluble residue contains P which is liberated as phosphate by the usual HIO_4 technique. It also sometimes contains other forms of P, e.g. acid-hydrolysable P. In other cases (e.g. 'unidentified lipid 1', see below) it does not contain apparent glycerophosphate (HIO_4-liberated phosphate).

The fatty acids and the insoluble debris, but not the unsaponifiable matter, resulting from alkali hydrolysis of 'unidentified lipid 2' all contained some P. Allowing for this, and for the total P found in the clear aqueous phase before evaporation, vacuum drying and re-solution, only 80% of the original 3·8% P in the fraction could be found after alkaline hydrolysis. This clear aqueous layer contained P liberated after HIO_4 oxidation amounting to 2·3% of the original lipid, i.e. there was an apparent glycerophosphate content equivalent to 2·3% P. After the vacuum-drying procedure and removal of the insoluble residue, the apparent glycerophosphate P had fallen to 1·7%. (In this particular case, direct estimation of HIO_4-liberated phosphate was not made on the insoluble residue, but fraction Y[1], which formed over half of 'unidentified lipid 2', gave a similar residue which with HIO_4 yielded phosphate equivalent to 1·6% on the original lipid.)

It has now been further noted that, with the present lipids at least, not all the phosphate produced with HIO_4 in the method used (Olley & Blewett, 1950) represents glycerophosphate. On addition of $Ba(OH)_2$ to the aqueous solution of the alkaline hydrolysate, until pink to phenolphthalein, a precipitate was obtained. Barium glycerophosphate is soluble in water. After removal of the precipitate, glycerophosphate estimation on 'unidentified lipid 2' fell from 2·3 to 1·5%. In our previous paper (Lovern & Olley, 1953), glycerophosphate refers to the total phosphorus estimated with HIO_4 after removal of the insoluble residue. In the present paper a distinction is drawn between true glycerophosphate and 'apparent' glycerophosphate, the latter including the material giving an insoluble Barium salt. However, apart from 'unidentified lipid 2', all hydrolysates had been vacuum dried and water-insoluble residues discarded. Presumably, therefore, apart from this fraction, and also 'unidentified lipid 1' where the insoluble residue was found to be free from 'apparent' glycerophosphate, all 'apparent' glycerophosphate figures quoted may be too low. We have no reason to suspect loss of true glycerophosphate in the insoluble residue, which possibly consists of insoluble salts (Ca, Mg, etc.) of the 'false' glycerophosphoric acid.

EXPERIMENTAL

Counter-current distribution

Fraction *C* (112 g.) was submitted to counter-current distribution between light petroleum (b.p. 40–60°) and 85% (v/v) ethanol, in a series of thirty-three flasks, using 550 ml. of each phase, in the manner described previously (Lovern & Olley, 1953). After eleven batches of ethanol had been passed right through the series of flasks, the falling yield per batch and a change in the appearance of the recovered lipid suggested discontinuing the procedure. Further batches of ethanol were only transferred as far as possible down the series of flasks, without running them off from the end (flask 33), until it became obvious from the appearance of the ethanol phase that the latest batch was only transferring traces of lipid from flask to flask. This happened when flasks 18–33 contained both phases, and flasks 1–17 only the light petroleum phase. The entire contents of each flask were then recovered as a single fraction. The yield curve is shown in Fig. 1, the sequence of events, as in previous work, being arranged according to tendency towards one phase or the other. Thus the first eleven batches of ethanol yielded fractions 1–11, and the contents of flasks 33–1, in that order, yielded fractions 12–44. Dotted vertical lines indicate where the curve is really discontinuous.

The shape of the curve for fractions 1–11 indicates the presence of a series of overlapping fractions. In an effort to separate these, the fractions were added to the first eleven flasks in a new series of twenty-two flasks, each containing 800 ml. of light petroleum, after which batches of 85% (v/v) ethanol, each of 150 ml., were passed through the series. The use of a larger ratio of petroleum to alcohol should distribute the lipids more widely. The results of this redistribution are shown in Fig. 2.

Sixteen batches of ethanol were passed right through the series of flasks (fractions *S* 1–16, *S* = subfraction) and then the petroleum phase in each flask was evaporated to yield fractions *S* 17–38 (flasks 22–1, in that order). Fig. 2 shows clearly how complex was the material represented by fractions 1–11 of Fig. 1.

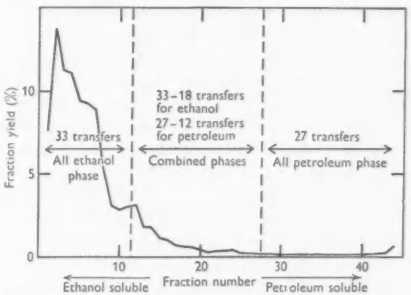

Fig. 1. Counter-current distribution of the acetone-insoluble lipids (fraction *C*) of an acetone extract of haddock flesh. Solvents, light petroleum (b.p. 40–60°) and 85% (v/v) ethanol. Phase ratio, 1:1. Curve passes through experimental value for every fraction.

Pooling of fractions

Fractions were combined according to physical appearance, shape of the distribution curves, and analytical data. Thus fractions *S* 1–5, which were all choline-rich (of the order of 10%), were combined to give a first 'mainly lecithin' fraction (29·2 g.), and fractions *S* 6–27 were similarly combined to give another choline-rich fraction (53·8 g.), differing from the first one in its distribution behaviour. Choline continued at a level of approximately 10% to fraction *S* 24, but fell to about 5% at fraction *S* 27, and to 2·5% at fraction *S* 30, thereafter becoming negligible. The physical appearance of the fractions altered appreciably between fractions *S* 27 and *S* 28, although there was a general resemblance to phospholipin until fraction *S* 34, from which point onwards fractions were increasingly wax-like in appearance. Fractions *S* 28–38 (12·7 g.) were pooled to give a product corresponding to the last main component of Fig. 2, virtually choline-free.

Fractions 12–25 of Fig. 1 resembled phospholipins in appearance, and were combined (13·5 g.).

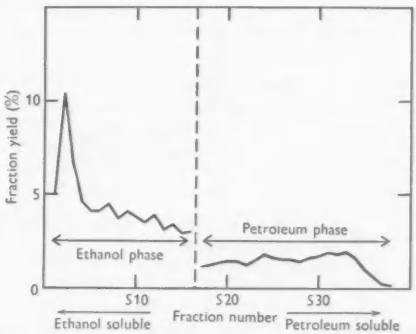

Fig. 2. Counter-current distribution of fractions 1–11 in Fig. 1 between light petroleum and 85% ethanol. Phase ratio, 8:1·5. Curve passes through experimental value for every fraction.

Fractions 26–41 resembled viscous waxes and were combined (1·6 g.). Fractions 42 and 43 were also viscous waxes, but somewhat darker, and were combined as another fraction (0·4 g.). Finally fraction 44 (0·8 g.) was an almost black substance, insoluble in light petroleum but soluble in chloroform apart from a trace of residue.

These manipulations are illustrated in Fig. 3, in which all weights are corrected for manipulative and analytical losses. Some analytical data on the pooled fractions are given in Table 1.

Purification of lecithin fractions

Fractions *S* 1–5 and *S* 6–27 were subjected to further fractionation by crystallization from a 10% (w/v) solution in absolute ethanol at −30°, according to Sinclair's (1948) method for the purification of lecithin.

Table 1. *Analytical data on lipid fractions*

(All values % constituent in fraction except N:P ratio which is atomic ratio.)

Fraction	N	P	N:P	Choline	Ethanolamine*	Plasmal
S 1–5	2·4	3·5	1·5	9·6	0·5	Nil†
S 6–27	1·8	3·9	1·0	7·8	0·7	0·4†
S 28–38	1·5	3·5	0·95	0·6	1·8	0·8†
12–25	1·4	2·9	1·1	Negligible	1·6	3·3‡
26–41	1·2	1·5	1·8	Negligible	0·3	4·2‡
42–43	1·5	2·2	1·5	Negligible	0·6	6·9‡
44	2·2	3·7	1·3	Negligible	0·6	7·6‡

* Only negligible amounts of serine in all fractions.
† Colorimetric estimation.
‡ Gravimetric estimation.

Fraction S 1–5. This was not entirely soluble even in warm ethanol (at approx. 40°). It was, therefore, first diluted to 5% (w/v) and filtered at 0°, giving 5·9 g. of a brown, salt-like residue (fraction *X*). cipitate of 2·1 g. (fraction X^2) and leaving 3·8 g. (fraction X^1) in solution.

Fraction X^1 contained 3·1% N and 1·5% P (N:P = 4·6) and on hydrolysis with 0·5N-ethanolic

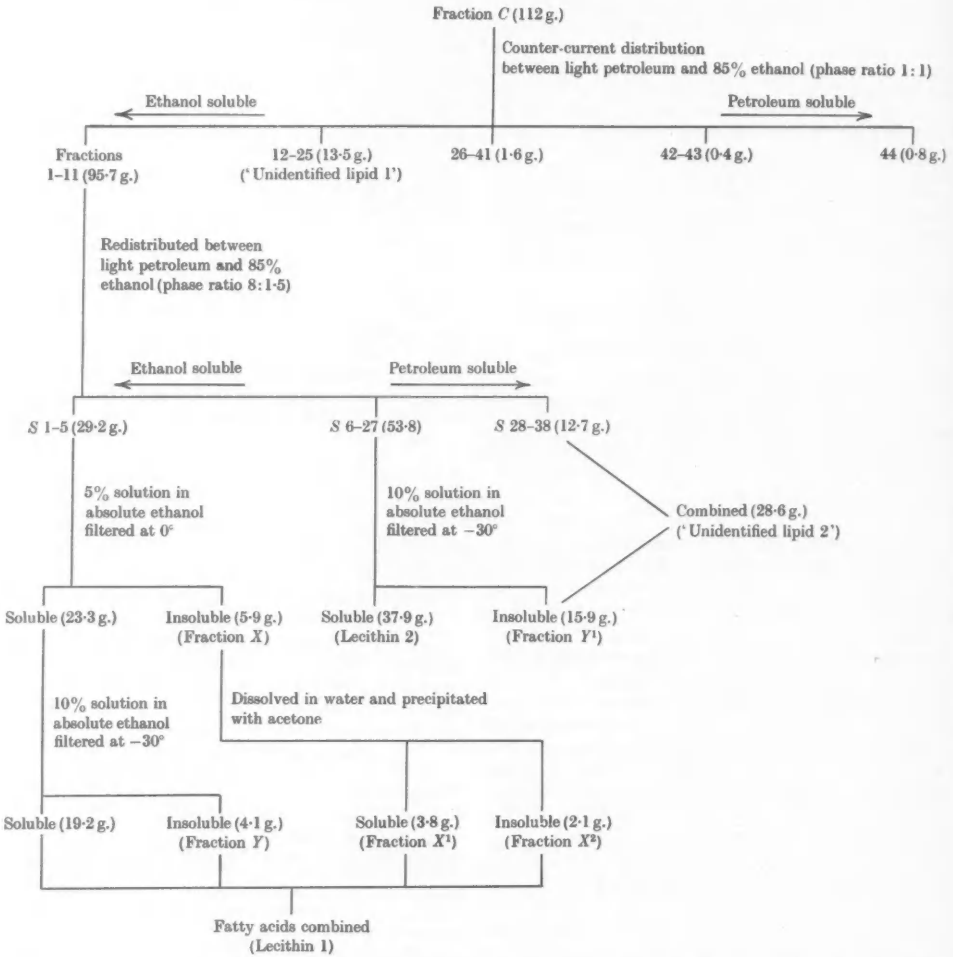
Fraction C (112 g.)
Counter-current distribution between light petroleum and 85% ethanol (phase ratio 1:1)
Ethanol soluble
Petroleum soluble
Fractions 1–11 (95·7 g.)
12–25 (13·5 g.) ('Unidentified lipid 1')
26–41 (1·6 g.)
42–43 (0·4 g.)
44 (0·8 g.)
Redistributed between light petroleum and 85% ethanol (phase ratio 8:1·5)
Ethanol soluble
Petroleum soluble
S 1–5 (29·2 g.)
S 6–27 (53·8)
S 28–38 (12·7 g.)
5% solution in absolute ethanol filtered at 0°
10% solution in absolute ethanol filtered at −30°
Combined (28·6 g.) ('Unidentified lipid 2')
Soluble (23·3 g.)
Insoluble (5·9 g.) (Fraction X)
Soluble (37·9 g.) (Lecithin 2)
Insoluble (15·9 g.) (Fraction Y¹)
10% solution in absolute ethanol filtered at −30°
Dissolved in water and precipitated with acetone
Soluble (19·2 g.)
Insoluble (4·1 g.) (Fraction Y)
Soluble (3·8 g.) (Fraction X¹)
Insoluble (2·1 g.) (Fraction X²)
Fatty acids combined (Lecithin 1)

Fig. 3. Fractionation scheme for the acetone-insoluble lipids present in the crude acetone extract of haddock flesh. All weights have been corrected for analytical and manipulative losses.

After concentration to 10%, the solution was held overnight at −30° when it yielded 19·2 g. of soluble lipid (a purified lecithin) and 4·1 g. of insoluble lipid (fraction *Y*). Fraction *X* was insoluble in ether, but soluble in $CHCl_3$ and readily soluble in water. It contained 3·2% choline. It was dissolved in 100 ml. water and 200 ml. acetone were added, giving a pre-

KOH yielded 9·4% fatty acids and 0·7% unsaponifiable matter. It was clearly mainly non-lipid material. Fraction X^2 contained 5·5% choline and 2·1% glycerophosphate P. On hydrolysis it yielded 27·3% fatty acids and 9·1% unsaponifiable matter. This also was a mainly non-lipid fraction, but since the total fatty-acid content of the original

fraction X (15·7 %) is in close agreement with that equivalent to the 3·2 % of choline (= 15·8 %, assuming a lecithin with an average fatty-acid chain of 19 C atoms), the fatty acids recovered from fractions X^1 and X^2 were included with those derived from the main lecithin fractions of S 1–5 as shown in Fig. 3.

The glycerophosphate P value given above for fraction X^2 has been corrected by Ba precipitation as described under analytical methods. Total 'apparent' glycerophosphate P was 2·4 %. The 5·5 % of choline in fraction X^2 is equivalent to 1·4 % of glycerophosphate P on the lecithin formula. Since the choline and fatty-acid contents are in good agreement, it would appear that fraction X^2 contains about 0·7 % of non-lipid glycerophosphoric acid.

The purified lecithin from S 1–5 contained only 10·2 % choline, the process, therefore, having been relatively ineffective. On hydrolysis with 0·5N-ethanolic KOH it yielded 40·3 % fatty acids, 2·4 % unsaponifiable matter and 2·4 % glycerophosphate P. The glycerophosphate is equivalent to 9·4 % choline and 46·2 % fatty acids (C_{19} average), and the choline content is equivalent to 49·3 % fatty acids. This fraction may well have contained both glycerophosphoric acid and choline in non-lipid combination. In other experiments with a mixture of fractions B' and C (Lovern & Olley, 1953), removal of non-lipid impurities by the method of MacLean (1912) indicated the presence of free choline, detected by paper chromatography. The fraction still contains about 40 % of non-lipid impurities, based on the fatty-acid content. The small amount of ethanolamine (0·5 %), equivalent to nearly twice its weight of choline, in the total fraction S 1–5 was not followed through the Sinclair fractionation, but its presence still further increases the excess of phospholipin bases over the glycerophosphate and fatty-acid figures.

Fraction Y was also predominantly a lecithin fraction, since it contained 8·6 % choline, 2·1 % glycerophosphate P (3·5 % total 'apparent' glycerophosphate P before Ba precipitation) and yielded 44·1 % fatty acids on hydrolysis. These values are all in good agreement for lecithin. Hydrolysis also yielded 4·7 % of unsaponifiable matter. Fraction Y would thus appear to be essentially lecithin contaminated with about 40 % of mainly non-lipid impurities.

The fatty acid from the three subfractions of S 1–5 were combined as already described to give the fatty acids of 'lecithin 1'. The unsaponifiable matter from these same subfractions was likewise combined. It contained 0·6 % N, no P, had an iodine value of 155·7 and after acetylation a saponification equivalent of 306·6. It would thus appear to consist mainly of higher aliphatic alcohols, there being no evidence from fatty-acid yields that these were originally present as wax esters.

Fraction S 6–27. This was entirely soluble in warm ethanol (approx. 40°) but at room temperature (about 15°) the solution deposited considerable amounts of a thick gum. Without separating this, the solution was cooled to −30° overnight and filtered, yielding 37·9 g. of soluble material (described as 'lecithin 2') and 15·9 g. of insoluble material, fraction Y^1.

Lecithin 2 contained 10·0 % choline, compared with the 7·8 % of the unfractionated S 6–27, 0·7 % ethanolamine and 2·7 % glycerophosphate P. On hydrolysis it yielded 64·2 % fatty acids and 1·7 % unsaponifiable matter. The choline content is equivalent to 2·6 % glycerophosphate P but to only about 50 % fatty acids. The ethanolamine can account for 7·3 % fatty acids and 0·4 % glycerophosphate P. The unsaponifiable matter (1·9 % N, no P) had an iodine value of 102·4 and the acetylated material a saponification equivalent of 360·8. It again appeared to consist essentially of higher alcohols. Even if it were present as fatty-acid esters, it cannot account for more than about 2 % of fatty acids, leaving some 5 % of fatty acids present as unidentified lipid. There is probably also some non-lipid impurity, but the amount is much less than in 'lecithin 1', probably of the order of 10 %, assuming all the fatty acid present in lipids of the phospholipin type.

Fraction Y^1 contained only 2·2 % choline, 0·7 % ethanolamine, 1·5 % N and 4·0 % P. Both on the basis of its position in the fractionation scheme, and on the above analytical data, it was combined with fraction S 28–38, as shown in Fig. 3.

Further examination of unidentified lipids

'*Unidentified lipid* 1.' Fraction 12–25 contained no choline or serine, 1·6 % ethanolamine, but plenty of nitrogen and phosphorus (Table 1) and resembled phospholipins in appearance. It contained 1·8 % total 'apparent' glycerophosphate P and 0·3 % acid-hydrolysable P, but the effect of Ba precipitation had not been noted when this fraction was examined. The ethanolamine is equivalent to 0·9 % glycerophosphate P. It was hydrolysed by refluxing for 2 hr. with 0·5N-ethanolic KOH. Unsaponifiable matter (7·0 %) was extracted and the fatty acids and plasmals separated by the method of Feulgen *et al.* (1929). The plasmal fraction (3·3 %) had an iodine value of 117·3 and the unsaponifiable matter (other than the plasmals) an iodine value of 92·3 and, after acetylation, a saponification equivalent of 587. This latter is higher than the expected value for higher aliphatic alcohols (about 300), and suggests a mixture of alcohols and hydrocarbons (cf. Lovern & Olley, 1953). The yield of fatty acids (53·1 %) is approximately what might be expected

from a phospholipin contaminated with small amounts of other lipids (represented by the unsaponifiable matter and the plasmal), but no known phospholipin base could be found apart from ethanolamine, half of which is equivalent to the plasmal present, the remainder accounting for only 8·4 % of fatty acids. Inositol could not be detected in paper chromatograms of the water-soluble constituents of acid hydrolysis. Alkaline hydrolysates did show by paper chromatography an amino compound in addition to ethanolamine, staining with ninhydrin, but probably present only in amount similar to the ethanolamine. There was also a pronounced spot corresponding to the phosphomolybdic acid-fixing base reported in the previous paper (Lovern & Olley, 1953). An attempt is being made to isolate this base, which has been found in variable amount in most of our phospholipin fractions. In contrast to choline, it does not give a water-insoluble reineckate. For the present this fraction 12–25 has been designated 'unidentified lipid 1', and it is probably of a phospholipin nature. The available glycerophosphate over that equivalent to the ethanolamine only corresponds to about 15 % fatty acids if the 'unidentified' lipid is based on the general lecithin pattern, whereas fatty acids attributable to 'unidentified lipid' total 41 %.

'*Unidentified lipid* 2'. Fractions Y^1 and *S* 28–38 were combined to give a fraction designated 'unidentified lipid 2', which appeared to be generally similar to 'unidentified lipid 1'. The combined material contained 1·6 % N and 3·8 % P (N:P = 0·93), 1·5 % choline, 1·2 % ethanolamine, negligible serine (paper chromatography), 1·5 % glycerophosphate P, 2·3 % total 'apparent' glycerophosphate P and 0·1 % acid-hydrolysable P. Hydrolysis with 0·5 N-ethanolic KOH yielded 3·3 % unsaponifiable matter (iodine value 105·1, saponification equivalent of acetates 300·3, presumably essentially higher aliphatic alcohol, containing, however, 1·6 % N but no P) and 52·9 % fatty acids (0·2 % N, 0·3 % P). There was also obtained on acidification of the alkaline hydrolysate 1·0 % of black debris, insoluble in both water and ether. This material contained 3·2 % N and 0·5 % P.

The choline and ethanolamine account for 7·4 and 12·6 % fatty acids respectively. Assuming 3·3 % fatty acids combined as waxes with the unsaponifiable matter, there remains 29·6 % fatty acids present as unidentified lipid. This would require 1·5 % glycerophosphate P if this lipid is structurally akin to lecithin, whereas the choline and ethanolamine present require 1·0 % P, leaving only 0·5 % glycerophosphate P available for the unidentified lipid. There is, however, approximately enough total 'apparent' glycerophosphate P available, but by analogy with 'unidentified lipid 1', where there was a lack even of total 'apparent' glycerophosphate, it would seem that the ratio of fatty acid to glycerophosphate in the unidentified lipid must be higher than in lecithin. Paper chromatography revealed the presence of the phosphomolybdate-reacting base discussed above, and also gave six spots, in addition to ethanolamine, staining with ninhydrin, four of which were more prominent than the other two, but none as marked as the ethanolamine spot. No inositol could be detected.

Later fractions. Fraction 26–41, in addition to the information given in Table 1, contained 0·9 % 'apparent' glycerophosphate P (true glycerophosphate not determined), and 0·3 % acid-hydrolysable P. After the customary alkaline hydrolysis it yielded 44·5 % unsaponifiable matter, 22·5 % fatty acids and 4·2 % plasmal. These substances had iodine values of 84·0, 168·5 and 67·3, respectively, and the acetylated unsaponifiable matter had a saponification equivalent of 1085, hence consisting largely of non-alcoholic lipid, probably hydrocarbon. A paper chromatogram of the water-soluble hydrolytic products closely resembled that of fraction 12–25.

Fraction 42–43 was apparently very similar to fraction 26–41, but contained more total N and P (Table 1). It contained 1·1 % 'apparent' glycerophosphate P and 0·6 % acid-hydrolysable P. Alkaline hydrolysis yielded 35·2 % unsaponifiable matter, 20·4 % fatty acids and 6·9 % plasmal, with iodine values of 56·4, 115·1 and 67·9, respectively. The acetylated unsaponifiable matter had a saponification equivalent of 1804, thus again containing a proportion of hydrocarbon material.

Fraction 44 contained still more total N and P than the previous two fractions, the phosphorus increasing more rapidly than the nitrogen (steadily falling N:P ratio). 'Apparent' glycerophosphate P was 1·2 %, acid-hydrolysable P 1·1 %. The steady rise in acid-hydrolysable P over the last three groups of fractions is interesting. This may well have represented some form of sugar phosphate, since acid hydrolysates of several fractions, tested with an ammoniacal silver nitrate spray, gave a spot which was not inositol, and which was possibly a sugar, especially since it also reacted with Novellie's (1950) sugar reagent (yellow spot). Hydrolysis yielded 16·5 % unsaponifiable matter, 25·8 % fatty acids and 7·6 % plasmal, with iodine values of 57·6, 211·8 and 70·5, respectively. The acetylated unsaponifiable matter had a saponification equivalent of 987.

Analysis of fatty acids

Sufficient material was available for detailed ester-distillation analyses of the fatty acids from lecithins 1 and 2, and from 'unidentified' lipids 1 and 2. These results are given in Table 2, together with the earlier analyses of lecithins *A* and *B'* (Lovern & Olley, 1953) for comparison.

Table 2. *Composition of the fatty acids of certain lipids of haddock flesh*

(All weights as % of total acids. Figures in brackets indicate average unsaturation in hydrogen atoms.)

	Saturated acids			Unsaturated acids		
Lipid	C_{16}	C_{18}	C_{20}	C_{18}	C_{20}	C_{22}
Lecithin *A**	13·5	5·7	2·9	12·6 (−5·4)	33·7 (−8·0)	27·3 (−12·0)
Lecithin *B′*	16·4	4·0	2·1	5·4 (−5·5)	42·2 (−8·0)	29·9 (−11·0)
Lecithin 1 (*C*)†	20·4	13·5	—	21·5 (−4·0)	28·5 (−7·0)	13·4 (−10·4)
Lecithin 2 (*C*)	22·7	4·8	0·8	16·1 (−3·3)	34·3 (−7·4)	21·3 (−9·7)
Unidentified 1 (*C*)	4·8	17·8	3·5	7·2 (−4·6)	46·1 (−7·2)	20·6 (−12·0)
Unidentified 2 (*C*)	6·1	17·9	2·2	8·4 (−2·0)	39·9 (−4·6)	25·5 (−11·1)

* Plus 4·3% C_{24} unsaturated acids.

† Plus 2·7% unsaturated (−4·0) C_{16} acids.

Table 3. *Components of pooled subfractions and total fraction C of the acetone extract of haddock flesh* (%)

Fraction	Wt. (g.)	Lecithin (*a*)	Phosphatidyl ethanolamine (*b*)	Unidentified lipid (*c*)	Plasmalogens (*d*)	Waxes and alcohols (*e*)	Hydrocarbons (*f*)	Non-lipids (*g*)
*S*1–5 (lecithin 1)	29·2	45·9	4·1	—	—	3·1	—	46·9
*S*6–27 'purified' lecithin (lecithin 2)	37·9	69·0	9·1	7·3	—	3·4	—	11·2
*S*28–38 + *Y*[1] (unidentified lipid 2)	28·6	10·3	15·6	41·5	—	6·6	—	26·0
12–25 (unidentified lipid 1)	13·5	—	10·4	57·6	5·9	7·2	3·4	15·5
26–41	1·6	—	—	14·3	7·5	24·6	32·2	21·4
42–43	0·4	—	—	20·4	12·3	11·6	29·4	26·3
44	0·8	—	—	29·1	13·5	10·0	11·5	35·9
Total fraction *C*	112	37·9	9·4	20·5	0·9	5·0	1·1	25·2

(*a*) Calculated from choline except in *S*1–5, where fatty-acid figure used. (*b*) From ethanolamine after allowing for ethanolamine present as plasmalogen. (*c*) From remaining fatty acid after allowing for lecithin, phosphatidyl ethanolamine and waxes. (*d*) From plasmal. (*e*) From unsaponifiable matter. Ratio of alcohol/hydrocarbon from sapon. equiv. after acetylation. Alcohols assumed esterified except in *S*1–5. (*f*) From sapon. equiv. of acetylated unsaponifiable matter. (*g*) By difference.

DISCUSSION

To facilitate comparison of fraction *C* with the fractions (*A* and *B′*) described in the preceding paper, a summation of the compositions of the various subfractions of fraction *C* described above is desirable. This is given in Table 3. The basis of the calculations employed has been outlined in the experimental section, but some features require further comment. It has been assumed, purely for the purpose of calculation in Table 3, that the unidentified lipid contains the same proportion of fatty acids as does lecithin. If this assumption is wrong, it will affect the relative proportions of both unidentified lipid and non-lipid, but not of the other constituents. Plasmalogens have been calculated from the plasmal figures as if all plasmalogen contained an ethanolamine base. In fact, in fractions 26–41, 42–43 and 44 there was less total ethanolamine present than that required for this assumption. The existence of plasmalogens not containing ethanolamine has been recorded (Klenk & Böhm, 1951; Klenk, 1952; Lovern, 1952). The relative proportions of higher aliphatic alcohols and non-alcoholic matter in the unsaponifiable matter have been calculated on the assumption that the acetates of the mixed alcohols would have a saponification equivalent of about 300. Apart from fractions *S*1–5, it has then been assumed that the alcohols were present as wax esters containing equivalent amounts of acid and alcohol. The non-alcoholic unsaponifiable matter is described as hydrocarbons, as in Lovern & Olley (1953).

Non-lipid contaminants

One of the most striking features of fraction *C*, particularly when compared with fractions *A* and *B′* (Lovern & Olley, 1953), is the progressive increase in

the proportion of non-lipid contaminants as the crude phospholipin material is supposedly purified by repeated reprecipitation with acetone. To facilitate comparison, the fraction described as 'unidentified material' in fraction *B'* has been recalculated as 'unidentified lipid', plasmalogen and 'non-lipid', on the basis used in the present paper. Fractions *A*, *B'* and *C* contained 5·4, 14·0 and 25·2 %, respectively, of non-lipid material. The persistence with which non-lipids accompany phospholipins in fractionation procedures is emphasized by the complete failure of counter-current distribution to effect any useful separation in this respect.

Unsaponifiable matter

Reprecipitation with acetone does not remove certain lipids which are assumed to be non-phospholipin in nature, e.g. waxes and hydrocarbons. These occurred in *A*, *B'* and *C*. The occurrence of alcoholic unsaponifiable matter in ox-brain and spinal-cord lipids extracted subsequently to acetone extraction of the tissue has been reported (Lovern, 1952). There is, of course, nothing specific about the acetone-precipitation process, e.g. Vaseline and the silicones are precipitated under the same conditions as phospholipins. However, whereas the hydrocarbons can be separated from the phospholipins by counter-current distribution (Table 3), this is not so for the aliphatic alcohols. It may be, as suggested in the previous paper, that some of this alcoholic material (e.g. that present in all main lecithin fractions of *A*, *B'* and *C*) is not present as simple waxes, but as part of a phospholipin-like substance. The occurrence of nitrogen in the unsaponifiable matter has been mentioned. This suggests the possible presence of sphingosine, in spite of the ready solubility of the original lipid in light petroleum and ether. However, paper chromatography of the unsaponifiable matter, in both butanol:acetic acid:water and phenol:ammonia systems failed to reveal the presence of sphingosine, although control samples of sphingosine were readily detected with both ninhydrin and phosphomolybdic acid. Sphingosine triacetate has a saponification equivalent of 158 and we have never found a saponification equivalent of the acetylated unsaponifiable matter lower than that expected (about 300) for the higher alcohols normally occurring in small quantities in fish oils, even in material from the main lecithin fractions, where hydrocarbons are almost certainly absent. The source of the nitrogen in the unsaponifiable matter is as obscure as that of the nitrogen in the fatty acid fraction and may represent a similar type of compound. We have confirmed the finding of earlier workers (cf. Lovern & Olley, 1953) that the nitrogen attached to the fatty acids cannot be removed by drastic hydrolysis and have also found this to be true for the phosphorus. Thus neither was split from the fatty acids when these were subjected to 24 hr. refluxing with 6N-hydrochloric acid. It would, therefore, appear most unlikely that the nitrogen and phosphorus represent incompletely hydrolysed phospholipin residues.

Unidentified lipid

Unidentified lipid was not found in fraction *A*, fraction *B'* contained 15·0 % of it (recalculated from data in Lovern & Olley, 1953) compared with the 20·5 % in fraction *C*. The fractionation data suggest that it may represent only one class of compound, since Table 3 shows that 98 % of the total amount was found in three groups of pooled fractions (*S* 6–38 and 12–25) representing roughly a continuous sequence in Fig. 1. Counter-current distribution between the solvents used can separate it reasonably well from lecithin, but there is little separation from phosphatidyl ethanolamine. The amount of available glycerophosphate compared to the fatty acids of the unidentified lipid is always less than that required for the classical glycerophosphatide structure (see p. 564), which suggests either that the lipid is not based on glycerophosphate at all or that there are additional hydroxyl or amino groups in the molecule available for combination with fatty acids. The finding of 'apparent' glycerophosphate, which was not acid-hydrolysable before but acid-hydrolysable after HIO_4 oxidation (resembling, therefore, glycerophosphoric acid), but having a water-insoluble barium salt, recalls the finding by McKibbin & Taylor (1952) of a polyglycerol phosphatide in dog-liver phospholipins.

Doubtful lipid constituents

The non-amino, phosphomolybdate-fixing base may be combined in a lipid molecule. Paper chromatography in butanol:acetic acid:water (40:10:50, v/v, at 20°) of a mixed lipid (fractions *B'* + *C*) largely freed from water-soluble non-lipid impurities by the method of MacLean (1912), showed only one spot staining with phosphomolybdic acid, this accompanying the phospholipins (R_F, 0·9). After alkaline hydrolysis of the same purified lipid, the now water-soluble bases showed two phosphomolybdate spots, one being choline (R_F, 0·23) and the other the new base (R_F, 0·1). The 'unidentified lipid' may perhaps contain this base.

Paper chromatography of hydrolysates of all fractions indicated the presence of a number of compounds, in addition to ethanolamine, staining pink with ninhydrin. These substances are possibly amino-acids, which are prominent constituents of the extractives of fish muscle and which are normally contaminants of crude phospholipin preparations. There is no evidence that they form part

of any phospholipin-like molecule, but it may be noted that counter-current distribution between these particular solvents is ineffective in separating them from the phospholipins.

There is always a possibility that certain other compounds present in the so-called 'non-lipid' fraction were present before hydrolysis in lipid form. The presence of acid-hydrolysable phosphorus has been mentioned. Many fractions, after hydrolysis with boiling 0·6N-sulphuric acid and paper chromatography of the water-soluble products, showed a spot reacting as a sugar with Novellie's (1950) reagent but showing only traces of reducing sugar with Somogyi's reagent (R_F, 0·38 in butanol:acetic acid:water (40:10:50, v/v, at 20°). In similar chromatography of the unhydrolysed fractions, this spot accompanied the phospholipin (R_F, 0·9). There is thus a suggestion that sugar phosphates are present as glycolipids. The great range of compounds contaminating crude phospholipins, even preparations completely soluble in light petroleum, is emphasized by the fact that crystals identified as sodium chloride separated from fraction *X*. Christensen (1939) has also found that phospholipins can carry sodium chloride into clear solution in light petroleum.

Plasmalogens

The iodine values of the crude plasmal mixtures (usually about 70, but reaching 117 in one case) indicate the presence of considerably greater proportions of unsaturated aldehydes than previously reported in plasmalogens (Leupold, 1950; Thannhauser, Boncoddo & Schmidt, 1951; Klenk, Stoffel & Eggers, 1952), which is perhaps not unexpected because the lipids of fish are generally more unsaturated than those of other vertebrates.

Fatty acids

The fatty-acid data in Table 2 indicate a difference between the acetone-soluble and the acetone-insoluble lecithins of this particular extract; a difference more marked than that noted by Hilditch & Shorland (1937) in the case of sheep-liver phosphatides, but agreeing with their finding that the acetone-soluble fraction is more unsaturated. For each group of unsaturated acids, the mean unsaturation is greater in lecithins *A* and *B'* than in lecithins 1*C* and 2*C*. Moreover, the proportions of the most unsaturated-acid groups (C_{20} and C_{22}) are reduced in the *C* lecithins, and the proportions of C_{18} acids and of saturated-acids increased. There are some smaller differences between the fatty-acid compositions of lecithins 1*C* and 2*C*, but the main difference is between acetone-soluble and acetone-insoluble groups.

The 'unidentified lipids' have a fatty-acid composition appreciably different from that of the lecithins, in particular containing more stearic acid at the expense of palmitic acid. The proportions of unsaturated acids show a general resemblance to those of the acetone-soluble lecithins. The degrees of average unsaturation in 'unidentified lipid 1' are similar to those of the lecithins, but in 'unidentified lipid 2' are markedly lower for the C_{18} and C_{20} acids. These lipid fractions, in particular 'unidentified lipid 2', represent mixed material (Table 3), but the change in the palmitic/stearic acid ratio is so marked in both cases that it seems likely to be a feature of the main component of the fractions, i.e. of the 'unidentified lipid'.

Little is known about the comparative fatty-acid composition of various phospholipins present in any particular tissue. Klenk (1952) has drawn attention to the probability that the fractionation procedures employed in isolating samples of fairly pure phospholipins will selectively modify the apparent composition of their fatty acids. Sinclair (1948) emphasizes the same thing in comparing methods of purifying lecithin. The present findings confirm this (comparison of the four lecithins and the two samples of 'unidentified' lipid). In comparing the compositions given in Table 2 it must be remembered that although figures are given to the nearest decimal point, the error on the small quantities of mixed fatty acids available (often less than 10 g.) is probably of the order of $\pm 10\%$ of the amount of any particular constituent. The higher stearic/palmitic ratio in unidentified lipid than in lecithin may be compared with a similar finding by Klenk (1952) for phosphatidyl serine and lecithin.

Counter-current pattern

The general counter-current distribution pattern follows that previously reported for the same solvent pair (Lovern, 1952). Lecithin is concentrated at the more polar (ethanol) end, followed by phosphatidyl ethanolamine. Plasmalogens and hydrocarbons are mainly concentrated towards the non-polar end. Waxes (or higher alcohols) are distributed over the entire system. For any one class of compound, there is again a suggestion (cf. Lovern & Olley, 1953) that the more unsaturated representatives are preferentially distributed towards the more polar end, although the position is often obscured by the presence of mixed classes of lipid. Thus, unsaponifiable matter other than plasmals has iodine values of 155·7, 102·4, 92·3, 84·0, 56·4 and 57·6 for fractions *S* 1–5, lecithin 2, 12–25, 26–41, 42–43 and 44 respectively (for fraction $Y^1 + S$ 28–38 the value is 105·1, but the precise position of this fraction in the sequence is not certain, it must be somewhere near lecithin 2). The relative proportions of alcoholic and hydrocarbon components vary, however. For plasmals, the iodine value was 117·3 for fraction 12–25, and about 70 in all following fractions.

Composition of total lipids extracted by acetone

The composition of the entire lipid fraction extracted by acetone from haddock flesh is given in Table 4 (summation of data on fractions *A*, *B'* and *C*). In spite of complete solubility in light petroleum, the second largest component is non-lipid matter. In view of the finding by Bähr & Wille (1931) of wide seasonal variation in the lecithin content of haddock flesh (really variation in ethanol:benzene-soluble P), it may be noted that much smaller variation is shown between three acetone extracts prepared at different seasons in the present work (Table 5). The evaluation of total phospholipins and total other lipids was derived by a combination of acetone precipitation and counter-current distribution of the acetone-soluble fraction (simplified from Lovern & Olley, 1953). The main phospholipin fraction of the acetone-soluble material was combined with the total acetone-insoluble material to give total phospholipins. Non-lipids would mostly accompany the phospholipin fraction. Those constituents of the non-phosphatidic lipid fraction given in Table 5 were determined directly. The constancy of the free fatty-acid figure is further evidence (cf. Lovern & Olley, 1953) that it is not a post-mortem artifact.

Table 4. *Composition of the total lipids extracted by acetone from haddock flesh*

	(%)
Lecithin	39·2
Unidentified lipid	12·5
Free cholesterol	6·4
Waxes	6·3
Free fatty acids	6·3
Phosphatidyl ethanolamine	4·7
Cholesterol esters	3·5
Triglycerides	2·4
Hydrocarbons	1·3
Plasmalogens	0·9
Non-lipids	16·5

Table 5. *Seasonal variation in amount of various lipids extracted by acetone from haddock flesh*

(All values as % of wet tissue.)

Sample	Crude phospholipin	Total non-phosphatidic lipid	Free fatty acid	Free cholesterol	Cholesterol esters	Triglycerides
Fish caught Aug.–Nov. 1951. Extracted in laboratory	0·43	0·10	0·036	0·036	0·020	0·014
Fish caught May 1952. Extracted at sea	0·44	0·08	0·034	N.D.	N.D.	N.D.
Fish caught Aug. 1952. Extracted at sea	0·61	0·15	0·034	0·048	0·034	0·011

N.D. Not determined.

SUMMARY

1. Some of the lipids present in a crude acetone extract of haddock flesh are practically insoluble in acetone at 0° after purification. This material has been fractionated by counter-current distribution between light petroleum and 85% (v/v) ethanol, certain products being further fractionated according to their solubility in absolute ethanol at −30°.

2. Two fractions consisting mainly of lecithin and two fractions consisting largely of an unidentified lipid were obtained in sufficient amount for detailed fatty-acid analyses. The lecithins differed from those isolated previously from the acetone-soluble fractions of the same total tissue extract, containing less $C_{20}+C_{22}$ and more C_{18} unsaturated acids. They also contained more total saturated acids. In addition, the various groups of unsaturated acids were less highly unsaturated. The fatty acids of the unidentified lipid differed from those of the lecithins mainly in containing more stearic acid at the expense of palmitic acid, and the two samples differed from each other in respect of unsaturation.

3. The unidentified lipid contained a higher ratio of fatty acids to glycerophosphate than the known glycerophosphatides. The presence in hydrolysates of a substance resembling glycerophosphoric acid but giving a barium salt insoluble in water, and of a non-amino base giving a water-insoluble phosphomolybdate, but different from choline, may afford clues to the composition of the unidentified lipid.

4. Minor lipid constituents included phosphatidyl ethanolamine, higher aliphatic alcohols and their esters, hydrocarbons or similar substances, and plasmalogens. These last were more unsaturated than any previously reported.

5. The material contained 25% of non-lipid impurities, more than the acetone-soluble fraction of the same original extract. Acetone precipitation does not, therefore, always afford purer phospholipin preparations. This non-lipid could not be removed by counter-current distribution as used, and every fraction was heavily contaminated. Various amino compounds, acid-hydrolysable phosphorus compounds and probably carbohydrates which were found in hydrolysates were possibly of non-lipid origin.

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Changes in the Water, Sodium and Potassium Content of Rat-liver Mitochondria during Metabolism

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Observations on the osmotic properties of mitochondria and their appearance in the phase-contrast and electron microscope suggest that these particles are surrounded by a membrane (Lazarow, 1943; Claude, 1944; Opie, 1948; Claude & Fullam, 1945; Zollinger, 1948; Dalton, Kahler, Kelly, Lloyd & Striebich, 1949). The existence of a selectively permeable membrane is borne out by the relative inaccessibility of the acid phosphatase of intact liver mitochondria to β-glycerophosphate, though the enzyme is readily demonstrable after lysis (Berthet, Berthet, Appelmans & Duve, 1951) and the fact that this phosphatase also becomes accessible after treatment with *Clostridium welchii* toxin suggests that the permeability is connected with the presence of lecithin (Datta & Macfarlane, unpublished).

Harman (1950) cast doubt on the existence of a semi-permeable membrane, since he found no selective penetration nor any concentration of cations in mitochondria present in cyclophorase preparation which had been oxidizing α-ketoglutarate. The demonstration of a concentration gradient, however, necessitates the separation of the mitochondria by centrifugation, and if during this time the metabolic process which is presumably necessary to maintain the gradient diminishes or stops it is probable that the gradient would be rapidly abolished. The conditions used by Harman were not optimal for the maintenance of metabolism; for example, centrifugation was carried out at 0°. We have carried out experiments with rat-liver mitochondria suspended in different media and subsequently centrifuged at room temperature, and have obtained evidence of the ability of the mitochondria to maintain a concentration of sodium and potassium ions above that of the surrounding medium; this ability appears to be linked with their capacity for oxidative phosphorylation and with the prevention of swelling.

After our experimental work was concluded, Bartley & Davies (1952) reported experiments demonstrating that mitochondria from sheep-kidney cortex could maintain concentration differences of inorganic and organic ions with the external fluid, in conditions similar to our own.

METHODS

Preparation of mitochondria. This was carried out in a room at 2–4° with chilled materials. Liver tissue, excised from rats immediately after death by bleeding out, was blotted with filter paper and weighed. Portions up to 5 g. at a time were ground in a precision-worked steel pressure mincer (Craigie, 1949), whose essential feature is a finely grooved plunger which operates in a closed cylinder; as the plunger is moved down the tissue is forced upwards through the grooves and efficiently pulped by a combination of cutting and shearing action. Much of the fibrous matter is caught in the grooves but almost all the pulp is forced to the top of the plunger and readily recovered by washing out with an appropriate liquid.

The liver pulp was suspended in 0·25 M-sucrose solution (Schneider, 1948) (10 ml./g. tissue), strained through fine muslin and centrifuged in 15 ml. pots at 2–4° for 30 min. at approximately 300 *g* to remove nuclei, red cells and unruptured liver cells. The supernatant was removed with a pipette and spun in a Servall angle centrifuge at approx. 7000 rev./min. for 30 min.; the supernatant and free-flowing layer of the deposit were removed, and the packed mitochondria resuspended in 0·25 M-sucrose or other liquids for washing or use as desired. The preparations were examined microscopically to check the absence of cells and nuclei. The separation of cell constituents by this technique, without washing, was fairly sharp judged by the succinoxidase activity, which is predominantly localized in the mitochondria (Hogeboom, Schneider & Pallade, 1948); about 10% of the original activity was found in the nuclear fraction, 65% in the mitochondrial suspension, and 5–10% in the supernatant containing the soluble constituents and microsomes.

Separation and analysis of mitochondria. After incubation of the mitochondria in various conditions described below, the mixtures (usually 24 ml.) were transferred to 50 ml. pots and spun in the Servall angle centrifuge at room temperature for 20 min. Longer spinning did not appreciably affect the degree of packing judged by the water content. The supernatant was removed, the last drops by draining and wiping with filter paper, and samples of the deposit immediately removed to tared silica crucibles with lids. After weighing and drying to constant weight at 105° to obtain the water content of the deposit (regarded as the mitochondrial water) the material was ashed overnight in an electric furnace at approx. 500°, and the ash dissolved in 10 ml. 0·3 N-H_2SO_4. Measured samples of the supernatant fluid were dried and ashed similarly. Glass-distilled water was used for the preparation of reagents and rinsing of apparatus.

The Na and K contents were estimated by the flame photometer (Spencer, 1950).

Other methods. Phosphorus estimations were made by a colorimetric method (Martland & Robison, 1926) to determine the following fractions: total P after ashing; apparent orthophosphate by direct estimation; acid-labile P by hydrolysis in N-HCl at 100° for 7·5 min. In several experiments the orthophosphate was also determined by estimation of the amount precipitable with $CaCl_2$ in 10% ethanol, to detect any phosphocreatine-like ester, but none was found.

Oxygen uptake was measured in a Warburg manometric apparatus, usually at 28° in air with 3·0 ml. reaction mixture, 0·5 ml. 20% (w/v) trichloroacetic acid (TCA) in the side arm and 0·3 ml. 2 N-NaOH in the centre cup. Readings were begun after 10 min. equilibration.

Materials. Cytochrome *c* was prepared according to Keilin & Hartree (1945). Crystalline synthetic adenosine 5′-phosphate (AMP) neutralized with NaOH was used.

RESULTS

Distribution of sodium and potassium in liver fractions

Preliminary experiments were made to establish the reproducibility of results with the methods used, and the range of values in different fractions. The results of two experiments are shown in Table 1. Two points are of interest. Firstly, only about 60% of the sodium but about 90% of the potassium in whole liver tissue (dried and ashed without grinding) was found in the liver pulp, indicating that a high proportion of the sodium is held in the connective tissue fibres. Secondly, the concentrations of sodium and potassium in the mitochondrial water were higher than in the supernatant liquid, i.e. a gradient had been maintained during the preparation. The supernatant is of course not a natural fluid, but a mixture of the extracellular and intracellular fluids diluted in sucrose solution.

Changes in the water, sodium and potassium content of mitochondria during oxidation of glutamate

It is well known that isolated mitochondria in presence of magnesium ions, inorganic phosphate and an adenine nucleotide such as adenosine triphosphoric acid (ATP) can oxidize metabolites of the Krebs tricarboxylic acid cycle with a coupled synthesis of high-energy phosphate compounds, the phenomenon being known as oxidative phosphorylation. It was expected that such a medium, which can support other synthetic activities, e.g. synthesis of *p*-aminohippuric acid (Kielley & Schneider, 1950), would be necessary for secretory activity. Kielley & Kielley (1951) showed that AMP can act as an external phosphate acceptor for the esterifica-

Table 1. *Distribution of sodium and potassium in rat-liver fractions*

(For details see text.)

	Na				K			
	mg./g. fresh tissue		m-equiv./l. water in fraction		mg./g. fresh tissue		m-equiv./l. water in fraction	
Sample	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
A, Fresh liver	1·46	1·36	90·6	84·5	4·21	4·47	154	164
B, Liver pulp	0·87	0·85	—	—	4·0	3·94	—	—
C, Fractions of *B*: nuclei and cells	0·036	0·052	5·8	—	0·15	0·27	14·4	—
Mitochondria	0·034	0·045	11·3	14·7	0·20	0·23	39·8	44·8
Supernatant	0·74	0·58	3·2	2·7	3·18	2·91	8·2	8·0

tion of inorganic phosphate to ATP and adenosine diphosphoric acid (ADP), and it was decided to use this simple system in the first place, with L-glutamate as substrate, and to consider the concentration of acid-labile P as a measure of the capacity of the system for oxidative phosphorylation.

The design of these experiments was limited by the time required for separating the mitochondria by centrifugation; with the Servall angle centrifuge used, holding 3 × 50 ml. pots, 20 min. was required for good packing. It was therefore not possible to follow the rapid initial changes on mixing the mitochondria with the medium, nor to take samples at less than 20 min. intervals. It is obvious that the oxygenation conditions are not optimal during centrifuging, and reversal may occur. Generally the experiments were arranged so that comparison could be made after a given time between the composition of mitochondria incubated in media identical save for the presence or absence of AMP, as well as between the mitochondria and the external medium.

Sucrose-low salt medium. Exp. 74. Unwashed mitochondria (10 ml., 17 mg. N) in 0·25 M-sucrose were added last to 20 ml. medium in each of five 250 ml. conical flasks (*A–E*), the final composition being: sodium L-glutamate, 0·02 M; $MgSO_4$, 0·002 M; potassium phosphate, pH 7·4, 0·01 M; cytochrome *c*, 2×10^{-5} M; calc. m-equiv./l.: Na, 20; K, 18. The flasks *A*, *B* and *C* also contained 0·001 M-AMP. Samples (3·0 ml.) were removed for estimation of O_2 uptake, and the open flasks rocked at 28°. After the desired time, a sample (3·0 ml.) was removed into 0·5 ml. cold 20 % (w/v) TCA, for estimation of acid-soluble phosphates. The remainder (24 ml.) was centrifuged for 20 min.: the supernatant was removed and measured, samples being taken for analysis, and the deposit of mitochondria weighed, dried and ashed.

The results are shown in Table 2. In the mixtures containing AMP there was a rapid esterification of orthophosphate to acid-labile P, and a decrease in the water content of the mitochondria followed by an increase in the sodium and potassium concentration. The acid-labile P, however, decreased as the incubation proceeded, and after 60 min. the water content was increasing and the sodium and potassium concentrations approaching that of the external fluid. The samples containing AMP in the manometric flasks were at this time still taking up oxygen at the initial rate (41 μl./mg. N/10 min.), but the uptake in the conditions of the experimental flasks may have been different. After 10 min. incubation there was an appreciable difference between the acid-labile P of the whole mixture and that of the supernatant subsequently separated by centrifuging, but at later stages the two values were practically the same. This suggested that, though some decrease in acid-labile P might take place during centrifuging, part of the initially formed acid-labile P was attached to the mitochondria, and that this part decreased during incubation more rapidly than that in the medium.

This was also indicated in another similar experiment, in which the acid-labile P was determined in the trichloroacetic acid filtrates from the whole mixture, and from the supernatant and deposited mitochondria. After incubation with AMP and L-glutamate for 10 min. at 28°, the acid-labile P in the whole mixture, the supernatant and deposit was 2·2, 1·8 and 0·11 mg., respectively; after 30 min. incubation the corresponding figures were 2·3, 2·2 and 0·03 mg.

Variation in potassium and sodium concentration of medium. Exp. 66. Unwashed mitochondria (10 ml., 19 mg. N) in 0·25 M-sucrose were added last to 20 ml. medium containing L-glutamate, $MgSO_4$, cytochrome *c* and AMP as before and varying amounts of Na and K as phosphate buffer, pH 7·4. Samples (3 ml.) were taken for the manometric flasks and the remainder incubated for 30 min. and the mitochondria separated and analysed as before.

The results, shown in Table 3, are similar to the previous experiment, but it may be noted that the rate of oxygen uptake was lower with the higher potassium concentration in the external medium. In other experiments in which the concentrations of sodium and potassium were varied by the addition of sodium chloride or potassium chloride, the rate of oxygen uptake was also lower when the potassium/sodium ratio of the suspending fluid was very high.

Table 2. *Sodium and potassium concentration in mitochondria oxidizing* L-*glutamate in a sucrose-low salt medium*

(The basal medium was the same in all flasks; flasks *A*, *B* and *C* contained also 0·001 M-AMP. The calculated concentrations were checked by analysis of supernatants from *A* and *D*. For further details see text.)

			Acid-labile P (mg./30 ml.)		Mitochondria			Supernatant	
						m-equiv./l. water		m-equiv./l. water	
Flask	AMP	Incubation (min.)	Suspension	Supernatant	Dry wt. (%)	Na	K	Na	K
A	+	10	1·32	0·93	20·2	29	29	26	18
B	+	30	0·78	0·75	20·5	52	68	—	—
C	+	60	0·39	0·40	19·0	24	31	—	—
D	0	10	0·06	0·06	12·5	26	30	22	16
E	0	30	0·06	0·06	11·6	22	22	—	—

Table 3. *Variation of sodium and potassium concentrations in external medium*

(For details see text.)

Flask	Phosphate concn. ($M \times 10^{-2}$)	O_2 uptake ($\mu l./10$ min./mg. N)	Mitochondria (*M*) Dry wt. (%)	Mitochondria (*M*) m-equiv./l. water Na	Mitochondria (*M*) m-equiv./l. water K	Supernatant (*S*) m-equiv./l. Na	Supernatant (*S*) m-equiv./l. K	Ratio of concn. *M*/*S* Na	Ratio of concn. *M*/*S* K
A	1	62	19·3	39	37	24	16	1·6	2·3
B	2·5	41	21·8	*	96	2	60	—	1·6
C	2·5	52	21·2	82	12	64	6	1·3	2·0

* Too low for estimation.

Table 4. *Sodium and potassium concentrations in mitochondria oxidizing* L-*glutamate in a potassium chloride medium*

(The basal medium was the same in all flasks; flasks *A*, *B*, *C* and *D* contained also 0·0015 M-AMP. The calculated concentrations were checked by analysis of supernatants from *A* and *E*. For further details see text.)

Flask	AMP	Time (min.)	Acid-labile P in supernatant (mg./30 ml.)	Mitochondria Dry wt. (%)	Mitochondria m-equiv./l. water Na	Mitochondria m-equiv./l. water K	Supernatant m-equiv./l. Na	Supernatant m-equiv./l. K
A	+	10	2·0	19·1	46	72	35	53
B	+	30	1·8	18·8	50	75	—	—
C	+	60	1·6	18·1	51	82	—	—
D	+	90	1·5	17·7	46	65	—	—
E	0	10	Nil	12·0	35	61	35	53
F	0	30	Nil	10·6	37	59	—	—

KCl *medium.* In other experiments, similar in design but using a medium like that of Kennedy & Lehninger (1949), the mitochondria were washed once in 0·25 M-sucrose and resuspended in 0·15 M-KCl. The final concentrations were: KCl, 0·05 M; sodium L-glutamate, 0·02 M; $MgSO_4$, 0·005 M; Na_2HPO_4-KH_2PO_4 (pH 7·4), 0·01 M; cytochrome *c*, 3×10^{-5} M; AMP (when present) 0·0015 M. Calc. Na, 39; K, 52 m-equiv./l.

The results of one such experiment are shown in Table 4, and are similar to those of Exp. 74.

The initial water content of mitochondria deposited from the sucrose solution was about 83%; on incubation with glutamate, but without AMP, there was usually no oxygen uptake, and the water content increased to 88–90% while the sodium and potassium approached that of the medium; occasionally with unwashed mitochondria there was a slight oxygen uptake, the concentration gradient was maintained and the water content was lower than usual in these controls, although no acid-labile P was detectable. This may have been due to traces of adenine nucleotides in the suspension.

Generally speaking, the concentrations of sodium and potassium in the mitochondria in presence of glutamate and AMP were greater than those in the medium or in mitochondria incubated without AMP, and the maintenance of this higher level appeared to be linked with a decrease in the water content and with the concentration of acid-labile P in the system, which in these experiments generally decreased as the incubation time was prolonged.

In Fig. 1, the water content of the separated mitochondria has been plotted against the amount of acid-labile P present in the mixture before centrifuging; the points are taken from samples obtained during the course of two experiments with each of two media. For either medium, there is clearly an association between the water content of

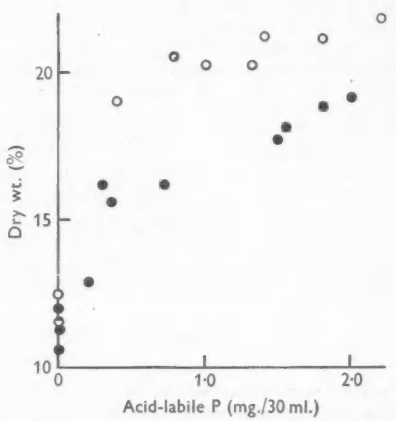

Fig. 1. Correlation between water content of mitochondria and the concentration of acid-labile P in the system. ○—○, Exps. 74 and 75, sucrose-low salt medium; ●—●, Exps. 85 and 88, KCl medium.

the mitochondria and the concentration of the acid-labile P in the system; the experiments illustrate the necessity of having an internal check, such as the level of esterified P, on the state of the mitochondrial sample analysed for electrolytes, even though the conditions are ostensibly suitable. It appears from the following experiments with dinitrophenol (DNP), however, that this association does not reside directly in the amount of external high-energy phosphate formed, but in some at present undefinable property of the mitochondria which is linked with their capacity (or potential capacity) for oxidative phosphorylation.

Effect of DNP on oxygen uptake and water content of mitochondria oxidizing L-*glutamate*

The effect on the swelling of mitochondria of DNP, which in general in the systems used abolishes the coupled phosphorylation and increases the rate of oxygen uptake, was examined in two experiments, in conditions similar to those in Table 2. Table 5 shows that in both experiments the rate of oxygen uptake was increased by DNP; in the first experiments the concentration used did not suppress the phosphorylation completely, but in the second experiment the acid-labile P was no greater than in the control without AMP. The water content of the mitochondria, however, was substantially the same in presence or absence of DNP, when AMP was also present; in absence of AMP the water content rose as usual.

Effect of adenylic acid on the agglutination and water content of mitochondria oxidizing succinate

It was noticed in the foregoing experiments that in the absence of AMP the mitochondria became agglutinated during incubation. This point was investigated further, using succinate as a substrate, as the oxidation of this substance *per se* does not require AMP.

Exp. 87. 1·0 ml. mitochondrial suspension in 0·25 M-sucrose (1·6 mg. N) was added last to 2·0 ml. medium in manometric flasks, the final concentrations being 0·02 M-sodium succinate; 0·005 M-$MgSO_4$; 2×10^{-5} M-cytochrome *c*; 0·001 M-AMP (when present); Na_2HPO_4-KH_2PO_4, pH 7·4 as indicated. The O_2 uptake at 28° was measured. Duplicate mixtures were kept at room temperature and smears made at intervals for microscopic examination.

The results for the lowest and highest phosphate concentrations are shown in Table 6. The oxygen uptake was initially considerably higher in the flasks without AMP, but decreased steadily and had almost ceased after 1 hr.; the oxygen uptake in presence of AMP decreased much more slowly and was proceeding at about 40% the initial rate after 80 min. Agglutination occurred within 10 min. in the mixtures with the lowest salt concentration and no AMP, and at each salt concentration was delayed in presence of AMP. These mixtures were not shaken, and it is possible that agglutination occurred more rapidly in the manometric flasks.

In another experiment the effect of AMP on the water content was determined.

Table 5. *Effect of dinitrophenol on water content of mitochondria*

(General conditions as in Exp. 74, see text.)

	AMP ($M \times 10^{-3}$)	DNP ($M \times 10^{-5}$)	Incubation time (min.)	O_2 uptake (μl./mg. N/10 min.)	Acid-labile P (mg.)	Mitochondrial dry wt. (%)
Exp. 75	1	0	10	39	1·8	21·1
	1	0	30	37	2·2	21·8
	1	2	10	50	1·0	20·2
	1	2	30	49	1·4	21·2
	0	0	10	0	0·2	12·9
	0	0	30	0	0	11·5
Exp. 76	2	0	15	41	2·8	20·9
	2	5	15	70	0·4	20·2
	0	0	15	0	0·4	13·9

Table 6. *Effect of AMP on oxidation of succinate and on agglutination in mitochondrial suspensions*

(Exp. 87, for details see text.)

Flask	Phosphate (M)	AMP ($M \times 10^{-3}$)	Rate of O_2 uptake (μl./mg. N/10 min.) at 10 min.	30 min.	60 min.	Agglutination at 10 min.	30 min.	60 min.
A	0·01	0	144	101	24	+	++	++
B	0·01	1	118	103	55	0	0	+
C	0·075	0	195	65	13	0	+	++
D	0·075	1	114	95	58	0	0	0

+, Microscopic; ++, macroscopic agglutination.

Mixtures were set up in two 250 ml. flasks *A* and *B* containing, in 30 ml. total vol.: 0·01 M-potassium phosphate, 0·01 M-$MgSO_4$, 2×10^{-5} M-cytochrome *c*, 10 ml. mitochondria in 0·25 M-sucrose, 0·01 M-sodium succinate. Flask *B* also contained 0·002 M-AMP. The flasks were rocked at 28° for 20 min., at which time O_2 was still being taken up in both the manometric samples, and the mitochondria separated and analysed as usual.

The water content was 88 % in *A* and 80 % in *B*, indicating that swelling had occurred in the absence of AMP, although oxidation was proceeding.

DISCUSSION

The experiments reported here show that, in contrast to the findings of Harman (1950), isolated mitochondria can maintain concentration differences of sodium and potassium ions between themselves and the suspending medium in certain conditions, i.e. they can do secretory work. This was demonstrated firstly by using a medium containing L-glutamate, inorganic phosphate, magnesium ions and adenosine 5′-phosphate (AMP), in which, as has been shown by other workers, the mitochondria can oxidize the substrate with a coupled esterification of inorganic phosphate to high-energy phosphate compounds such as ATP; and secondly, by maintaining this state of active metabolism as much as possible during the separation of the mitochondria for analysis by centrifuging at room temperature, instead of at 0°. The method has obvious limitations, particularly in assessing the absolute values of the concentrations which can be maintained, since reversal may occur during centrifuging, but it seems practicable for the direct study of the effect of specific conditions, such as adrenalectomy, or of specific substances upon the osmotic activity of the isolated mitochondria.

These preliminary experiments, which were made with rat-liver mitochondria, show that the maintenance of a concentration gradient and the prevention of swelling in hypotonic media are dependent upon the metabolic activity. When mitochondria were incubated with glutamate but without AMP, no oxidation occurred, the sodium and potassium concentrations of the mitochondria approached that of the medium and the water content rose to about 90 %; in these conditions the mitochondria agglutinated rather rapidly. In the presence of AMP, there was a concentration gradient, the water content decreased slightly from the initial value and was maintained at about 80 % and agglutination was deferred; in these experiments the rate of oxygen uptake often decreased on prolonged incubation and the acid-labile P also often decreased from an initial rapidly achieved maximum so the conditions were clearly not ideal.

In any particular system, as is shown in Fig. 1, there is some association between the level of acid-labile P and the degree of swelling, and the experiments using succinate as a substrate in the presence of AMP also indicate that the prevention of swelling is associated with the capacity of the system for oxidative phosphorylation, or more accurately, with the presence of an adenine nucleotide. On the other hand, in the presence of dinitrophenol, swelling was prevented although the level of external acid-labile P was practically nil. It is conceivable that the maintenance of selective permeability is dependent on the phosphorylation of some constituent of the membrane, that this constituent may be inactivated by incubation in unfavourable conditions and that it is in some way concerned in the phenomenon of oxidative phosphorylation. The facts observed may be related to the protective effect of adenine nucleotides on the 'ageing' of mitochondria incubated without substrate, noted by Kielley & Kielley (1951); on the other hand, 'ageing' may be due to the loss by diffusion of essential coenzymes from the mitochondria as our experiments show that swelling and equilibration with the medium occur rapidly in absence of AMP.

The ratios of internal/external concentration observed in the present experiments with rat-liver mitochondria, about 1·5–2 for sodium ions and 2–3 for potassium ions, are not high, compared, for instance, with the ratios for potassium in muscle and red blood cells. Bartley & Davies (1952) found in similar experiments with sheep-kidney mitochondria a high ratio (26) for sodium ions and also a marked concentration of fumarate. Apart from any function in the transport of ions peculiar to a particular tissue, it may well be an important consequence of the maintenance of a selective permeability that intermediary metabolites are kept in relatively high concentration in proximity to corresponding enzyme systems localized in the mitochondria.

SUMMARY

1. The ability of rat-liver mitochondria to do secretory work has been demonstrated by incubating them in a medium which would support oxidative phosphorylation and maintaining them, during separation for analysis, in a state of active metabolism by centrifuging at room temperature.

2. On incubation in a medium containing L-glutamate, magnesium ions, inorganic phosphate and adenosine 5′-phosphate (AMP), mitochondria maintained a higher concentration of sodium and potassium ions than that of the external fluid, and swelling was prevented.

3. In absence of AMP there was no concentration gradient, the water content rose from 80 to 90 % and agglutination readily occurred.

4. Swelling did not occur in the presence of AMP plus dinitrophenol, although oxidative phosphorylation was prevented.

5. It is concluded that the secretory action of mitochondria is dependent on active metabolism in the presence of an external adenine nucleotide and is linked with the capacity for oxidative phosphorylation in some way at present unknown.

We are indebted to Messrs Roche Products Ltd., for the gift of crystalline synthetic adenylic acid.

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Variations in the Ionic and Lactose Concentrations of Milk

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The ionic composition of cows' milk differs markedly from that of the blood serum; in milk the concentrations of sodium and chloride are lower than in blood, and the concentration of potassium is higher. This difference is greatest at the beginning of lactation, but later the ionic composition of milk tends towards that of blood: the sodium and chloride concentrations rise, while the potassium concentration falls. The lactose concentration of milk also falls as lactation advances. Milk samples from cows with mastitis show changes in composition similar to those found in advanced lactation. It has been shown that there is a relationship with a high correlation coefficient between the sodium and chloride concentrations in milk samples taken at any time in lactation, which can be expressed by a linear regression equation (Jones & Davies, 1935). There is a similar relationship between the lactose and chloride concentrations (Mathieu & Ferre, 1914).

In this paper the results are presented of a more detailed study than has been made previously of these changes in milk composition. First, the sodium, potassium and chloride concentrations in milk samples from thirty-eight cows in different stages of lactation were determined together with the sodium, potassium and chloride concentrations in blood serum samples from some of the same cows. From the results it is shown that there are relationships with high correlation coefficients between the potassium concentrations of these milk samples and their sodium and chloride concentrations which can be expressed by linear regression equations. These milk samples were all taken from separate cows with blood sera of slightly differing composition. The relation between the changes in milk composition and the composition of blood serum can be more clearly shown by analysing milk from the separate quarters of individual cows, since all four samples are formed from a common blood supply. Milk samples from the separate quarters of a cow are normally almost uniform in composition, but when a quarter is infected with mastitis organisms, the composition of its milk changes to an extent depending on the severity of infection. Secondly, therefore, the changes in milk composition were studied by analysing milk samples from the separate quarters of cows with mastitis, and blood samples from the same cows.

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EXPERIMENTAL

Sampling of blood and milk

The first series of milk samples were from thirty-eight healthy cows and were taken from the total milk yielded by each cow at a single milking. Since the concentrations of sodium, potassium and chloride in the blood serum vary little from cow to cow, blood samples were taken from only a few of the cows. They were drawn from the jugular vein immediately after collecting the milk, kept at 37° for 1 hr., and the serum then separated by centrifugation.

The milk samples (150 ml.) from the separate quarters were drawn at the beginning of normal milking. For this study it was necessary to find cows giving milk of different composition from each quarter, so a rapid survey was made of the quarter-samples from a large number of cows with mastitis by determining the chloride concentration of each. Seven of these cows were then selected for detailed analysis of their milk and blood. Immediately after milking, blood samples were taken and the serum separated as before.

Analytical methods

Sodium in milk was determined gravimetrically by the zinc uranyl acetate method (Barber & Kolthoff, 1928). Before precipitating the triple salt, the phosphate of milk, which would form a precipitate of uranyl phosphate with the reagent, and the protein and fat must be removed. Normally these are removed in two stages: protein and fat by wet ashing or precipitation, and phosphate by precipitation as zinc or calcium phosphate. For this work a more rapid and satisfactory procedure was devised in which the protein, fat and phosphate are removed in a single precipitation with uranyl acetate and acetic acid. Uranyl acetate has been previously used to remove phosphate from urine before sodium determination (Jendrassik & Halász, 1938), and also to remove protein and fat from milk before determining chloride (Kopatschek, 1922). Sodium in blood serum was also determined gravimetrically by the zinc uranyl acetate method, after removing proteins by precipitation with trichloroacetic acid.

Potassium in milk was determined gravimetrically by a sodium cobaltinitrite method (Piper, 1934), on the filtrate obtained after removing proteins and fat by precipitation with trichloroacetic acid. Potassium in blood serum was determined by an improved absorptiometric sodium cobaltinitrite method which has been published recently (Barry & Rowland, 1953).

Chloride in milk was determined by the method of Davies (1932), and chloride in blood serum by Whitehorn's application of the Volhard titration (Peters & Van Slyke, 1932).

Lactose was determined by a modification of the chloramine-T method of Hinton & Macara (1927).

RESULTS

Composition of the milk of individual cows

The relationships between the sodium and chloride, sodium and potassium, and potassium and chloride concentrations in thirty-eight milk samples from cows at various stages of lactation are shown graphically in Fig. 1, and the analyses which were made on blood serum from these cows are also plotted. The straight lines were drawn from linear regression equations, calculated from the milk analyses by the method of least squares. The regression equations, the standard errors of the regression coefficients, and the correlation coefficients, are shown in Table 1. For the components of a biological system the correlation coefficients are surprisingly high. A similar relationship between sodium and chloride has been shown before by Jones & Davies (1935), although our equation for the regression of sodium on chloride differs slightly from their equation of $Cl = 1{\cdot}24\ Na + 18{\cdot}09$. This equation has often been used for calculating the approximate sodium concentration in a milk sample from the more easily determined chloride. If the sodium, potassium or chloride concentration of a milk sample is known it is now possible, using the equations in Table 1, to calculate the approximate concentrations of the two other elements. These relationships, which were derived for normal lactation, also apply to mastitis milk as is shown below.

Composition of milk from the separate quarters of the cow

The relationships between the sodium, potassium and chloride concentrations in the quarter samples from a typical one of the seven cows which were selected because they gave milk of different composition from each quarter, are shown in Fig. 2, and the blood-serum analyses are also plotted. The correlations are similar to those found in the milk samples from individual cows but are much more precise, the points lying almost exactly on straight lines. The relationships between the lactose and the sodium, potassium and chloride concentrations in the same milk samples are also shown. The blood-serum analyses have been plotted assuming that the reducing-sugar concentration of the serum corresponds to 0·10% lactose (equivalent to 0·05% glucose which is approximately the normal value). Similar precise correlations were found in the quarter samples of the remaining six cows studied in this way, although the regression equations were slightly different from cow to cow; the points always fell amost exactly on straight lines, and the blood analyses lay in similar positions relative to the lines. Therefore, each cow has apparently its own regression equation depending on its blood composition, but since the blood composition varies only slightly from cow to cow, milk samples from a large number of cows give the almost linear relationships shown in Fig. 1.

DISCUSSION

Figs. 1 and 2 show that the composition of normal milk early in lactation lies near the point *A*, and that late in lactation or in mastitis it approaches *B*, thus

Table 1. *The correlation of sodium, potassium and chloride in the milk of cows at various stages of lactation*

Correlation between	Regression equations	Standard error of regression coefficients	Correlation coefficients
Na and Cl	Cl = 0·924 Na + 54·6	$5{\cdot}50 \times 10^{-3}$	0·98
	Na = 1·032 Cl − 52·6	$6{\cdot}17 \times 10^{-3}$	
Na and K	K = −0·570 Na + 183·5	$5{\cdot}00 \times 10^{-3}$	−0·95
	Na = −1·59 K + 298·8	$1{\cdot}39 \times 10^{-2}$	
K and Cl	Cl = −1·53 K + 339·5	$1{\cdot}067 \times 10^{-2}$	−0·97
	K = −0·613 Cl + 216·6	$4{\cdot}37 \times 10^{-3}$	

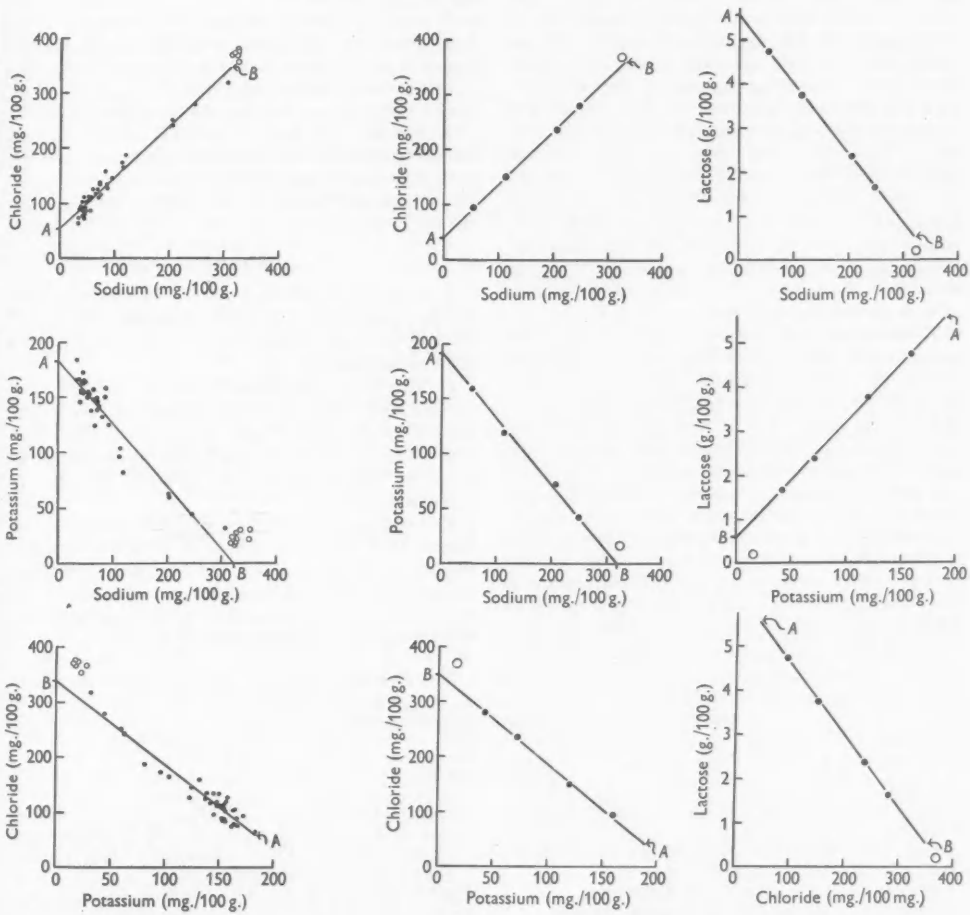

Fig. 1.

Fig. 2.

Fig. 1. Relationships between the Na, K and Cl concentrations in milk samples from individual cows and in blood plasma samples from some of the same cows. Milk composition is represented by solid circles and plasma composition by hollow circles.

Fig. 2. Relationships between the Na, K, Cl and lactose concentrations in milk samples from the separate quarters of a single cow and in the blood plasma of the same cow. Milk composition is represented by solid circles and plasma composition by hollow circles.

tending approximately towards the composition of blood serum. These changes can be considered as follows. Suppose two solutions whose compositions are represented by the points A and B (Figs. 1 and 2) are mixed in varying proportions; then the composition of any of these mixtures will also lie on the straight lines AB. Therefore, the composition of all milk samples could result from the mixing of a 'true milk' whose composition is constant and lies on AB near A, with a diluent whose composition is also constant and lies on AB near B; mixtures of 'true milk' and diluent in all proportions will lie on the straight lines AB. It can be seen that the diluent would differ slightly in composition from blood serum, since the serum analyses do not lie on the lines AB. The composition of the diluent cannot be calculated without knowing the concentration of one of its constituents. But since its potassium concentration cannot be less than zero, Figs. 1 and 2 show that its sodium and chloride concentrations during normal lactation and mastitis could not be greater than about 320 and 340 mg./100 g. respectively. The concentrations of sodium and chloride in the diluent would thus be slightly lower than in normal transudates.

There are two distinct mechanisms by which these variations in milk composition could be produced within the mammary gland. First, in advanced lactation and during mastitis, the milk secreted by the alveolar epithelial cells may change in composition; that is, the hypothetical mixing of 'true milk' and diluent occurs within the epithelial cells. This is the generally accepted mechanism (Turner, 1946). Secondly, throughout lactation and during mastitis, the epithelial cells may secrete a milk in which the concentrations of sodium, potassium, chloride and lactose are constant, and which, after secretion, is mixed with a diluent in which the concentration of these components is approximately the same as in blood serum. A similar mechanism has been suggested previously from other evidence (Davies, 1933; Peskett & Folley, 1933). The diluent, for example, might be a transudate from blood serum which continually filters into the lumen of the alveolus without passing through the epithelial cells; when the volume of milk secreted by the epithelial cells is depressed during late lactation or mastitis or certain artificial treatments (Turner, 1946), the proportion of transudate in the milk withdrawn from the mammary gland will rise.

There is too little evidence to decide which of these is the correct mechanism, but the following fact can be explained better by the second. The sodium, potassium and chloride concentrations in milk early in lactation are similar to those in most body cells: the potassium concentration is much higher than in blood serum and the sodium and chloride concentrations are lower (Shohl, 1939). The reason for this may be that the alveolar epithelial cells must secrete a fluid whose ionic composition is similar to that of their normal cell contents; if this is true, it would be expected that the ionic composition of this fluid would be maintained throughout the life of the cell.

SUMMARY

1. A study has been made of the variations in the sodium, potassium, chloride and lactose concentrations of milk and their relation to the composition of blood serum.

2. The linear regression equation relating the sodium and chloride concentrations in any milk sample is confirmed, and it is shown that the potassium and sodium, and the potassium and chloride concentrations are related by similar equations.

3. From analyses of blood and milk samples it is shown that the composition of all milk samples studied could result from the mixing, in varying proportions, of a milk similar to that produced early in lactation, with a fluid differing slightly in composition from blood serum.

This work was carried out during the tenure by one of us (J.M.B.) of a post-graduate scholarship from the Agricultural Research Council.

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The Biochemistry of the Nitrifying Organisms

4. THE RESPIRATION AND INTERMEDIARY METABOLISM OF *NITROSOMONAS*

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(*Received* 13 *November* 1952)

Nitrosomonas is an autotrophic micro-organism which depends for its free energy supply on the free energy released by the reaction

$$NH_4^+ + 1{\cdot}5\ O_2 = 2H^+ + NO_2^- + H_2O + 66\ 500\ \text{cal.}$$

Meyerhof (1917) found that the oxygen uptake of suspensions of the organism provided with ammonia agreed well with nitrite production in the suspensions. Bömecke (1939) established that the organisms had an oxygen uptake in the absence of ammonia; however, this endogenous respiration is very small and, although of considerable biochemical significance, is quantitatively negligible in ordinary respirometric studies in the presence of appreciable quantities of ammonia. The respiration of *Nitrosomonas* suspensions thus offers a very simple means of studying the metabolism of the organism since there is only one normal substrate and one end product; nevertheless, the studies of Meyerhof (1917) and Bömecke (1939, 1951) represent the bulk of the work that has been published in this field.

MATERIALS AND METHODS

The method used for the estimation of nitrite nitrogen (NO_2-N) has already been described (Lees, 1952*a*). NH_4-N was determined by the microdiffusion method, Conway (1950). Hydroxylamine was estimated by the method of Csaky (1949) in which the hydroxylamine is oxidized to nitrite by iodine and the nitrite so formed measured colorimetrically by the normal procedure.

Oxygen uptake was measured by the normal Warburg technique, using single side-arm vessels, 15–20 ml. total capacity, with 0·2 ml. 10% (w/v) KOH in the centre well. All experiments were carried out at 30°.

The organisms were grown as previously described (Lees, 1952*a*; Hofman & Lees, 1952) and the volume of the cultures was 400 ml. When the NO_2-N concentration of a culture had risen to approximately 100 μg./ml. the culture was centrifuged to separate the precipitate of carbonates and phosphates of Mg and Ca (the 'inorganic precipitate') initially present in the medium and to which the organisms adhere (Winogradsky, 1890; Meiklejohn, 1950; Lees, 1952*a*). The inorganic precipitate, with its adherent organisms, was then washed three times at the centrifuge with 200 ml. lots of distilled water; it was finally suspended in a suitable volume of distilled water and distributed by pipette into the Warburg vessels. The pH of the final suspension was that of the original medium, i.e. pH 8·5, the residual NO_2-N was about 0·2 μg./ml. and the residual NH_4-N about 1·0 μg./ml. (the NH_4-N was presumably mainly in the form of insoluble complex magnesium and calcium phosphates). It will be noted that the suspensions were washed with water and not with saline or any other iso-osmotic solution. Water washing was used because it was found that washing with NaCl, KCl, Na_2SO_4 or K_2SO_4 solutions, even at millimolar concentrations, caused loss of activity in the final suspension and that the loss of activity became progressively greater as the salt concentration was increased. This loss of activity was finally traced to dispersal of zooglea of the organisms in the salt solutions; no appreciable dispersal took place when water washing was employed.

For the preparation of washed suspensions at pH's different from pH 8·5, the cultures were first centrifuged to separate the inorganic precipitate, which was then suspended in 200 ml. distilled water and its pH (glass electrode) adjusted to the required final value by cautious dropwise addition of 0·1 N-HCl or 0·1 N-NaOH followed by vigorous shaking. This suspension was again centrifuged, the precipitate taken up in fresh distilled water, and the pH of the suspension readjusted to the required final value as before. After three such adjustments of pH the precipitate was taken up in buffer solution (0·01 M-KH_2PO_4 + 0·005 M-$Na_2B_4O_7$) which had already been adjusted to the required pH by means of 0·1 N-HCl or 0·1 N-NaOH. The suspension was then ready for use. This prolonged and cautious method of obtaining the required pH value in the final suspension ensured first that the slowly reached equilibrium between the suspension fluid and the carbonate of the inorganic precipitate was attained, and secondly that the organisms were never subjected to high and possibly injurious local concentrations of either acid or alkali during the course of pH adjustment.

The washed suspension from a single culture was usually distributed among eight to twelve Warburg vessels. Since the cultures were used when they had formed about 100 μg. NO_2-N/ml., it may be calculated from the results of Hofman & Lees (1952) that each vessel thus contained approximately 0·5–1·0 mg. dry weight of organism.

RESULTS

Table 1 shows that, within experimental error, washed suspensions of *Nitrosomonas* oxidize ammonia quantitatively to nitrite. Agreement between oxygen consumption and nitrite production was checked in all experiments; and such agreement is therefore to be inferred even where it is not specifically mentioned.

Table 1. *Ammonia oxidation, oxygen consumption, and nitrite formation by* Nitrosomonas

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in (approximately) 10^{-3}M-$(NH_4)_2SO_4$ and placed in the main compartments of the Warburg vessels. Temp. 30°; after temperature equilibration one vessel was removed and sampled for NH_4-N and NO_2-N. The taps of the remaining vessels were closed and the experiment continued, one vessel being removed every 20 min. and sampled for NH_4-N and NO_2-N as before.)

Time after vessel removed (min.)	O_2 uptake (μmoles)	NH_4 consumed (μmoles)	NO_2 formed (μmoles)	
			Calculated*	Observed
20	1·76	1·05	1·17	1·22
40	3·25	2·00	2·17	2·19
60	4·45	3·22	2·97	3·00
80	5·95	4·10	3·97	3·85
100	6·75	4·42	4·50	4·50
120	7·80	4·72	5·20	5·15

* Calculated from O_2 uptake.

The initial rate of oxygen consumption by the washed suspensions was reasonably linear over a wide range of ammonium sulphate concentrations. Curves corresponding to the extreme concentrations of ammonium sulphate tested are shown in Fig. 1. When almost all the ammonia initially present had been oxidized, the rate of oxygen consumption fell sharply and dropped practically to zero when ammonia oxidation was complete.

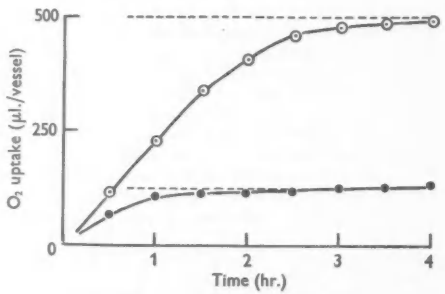

Fig. 1. Oxygen uptake by *Nitrosomonas* in the presence of different concentrations of ammonia. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas*. Ammonium sulphate (0·1 ml.) was added from the side arms after temperature equilibration to give initial $(NH_4)_2SO_4$ concentrations of $2{\cdot}5 \times 10^{-3}$M (curve —⊙—) or $6{\cdot}25 \times 10^{-4}$M (curve —●—) in the suspensions.

The influence of both the ammonia concentration and the pH of the suspension on the rates of oxygen uptake are shown in Figs. 2 and 3. The results of Meyerhof (1917) are included for comparison.

In previous incubation experiments with *Nitrosomonas* (Lees, 1952*a*) nitrite formation was markedly inhibited by allylthiourea. In the present work a similar inhibition of oxygen consumption by the allylthiourea was noted (Fig. 4). It was found that the inhibition of oxygen uptake caused by allylthiourea was completely abolished by washing the suspensions with water. The inhibition was not competitive with respect to ammonia over a wide range of ammonia concentrations (Table 2).

It has already been shown (Lees, 1952*b*) that hydroxylamine can be oxidized by *Nitrosomonas*, although the oxidation of hydroxylamine proceeds more slowly than that of ammonia if the hydroxylamine and ammonia are compared at nitrogen concentrations greater than about 2 μg./ml. It has now been found that although, as expected, hydroxylamine at 14 μg. nitrogen/ml. was oxidized more slowly than ammonia at the same nitrogen concentration, the oxidation of hydroxylamine remained virtually unaffected by concentrations of allylthiourea that completely inhibited oxidation of ammonia (Table 3). On the other hand, hydroxylamine oxidation was inhibited by hydrazine more strongly than was ammonia oxidation, and in the presence of hydrazine ammonia oxidation resulted in a small but definite accumulation of hydroxylamine (Table 4). The method used for hydroxylamine estimation involved a measurement of the difference in nitrite concentration before and after oxidation with iodine. It was therefore necessary, in order to demonstrate that without hydrazine no hydroxylamine accumulated during ammonia oxidation, to limit the amount of nitrite formed in these experiments and so minimize the possibility of masking small quantities of hydroxylamine by large quantities of nitrite. The experiments were therefore run for only a short period; as a consequence the oxygen uptakes were too small to be measured accurately and are omitted from Table 4.

DISCUSSION

The results of Table 1 show, as Meyerhof (1917) found, a quantitative agreement between oxygen uptake and nitrite formation in *Nitrosomonas* suspensions. The table also shows that nitrite formation closely paralleled ammonia disappearance; this fact proves that there could have been little, if any, accumulation of compounds intermediate between ammonia and nitrite during oxidation of the ammonia.

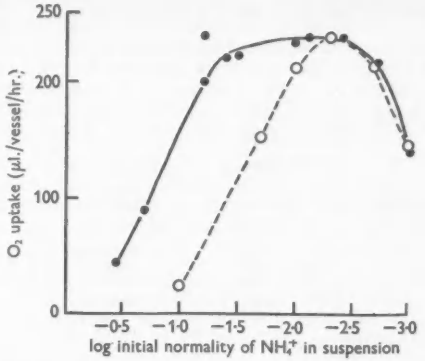

Fig. 2. The relation between ammonia concentration of the medium and rate of O_2 uptake by *Nitrosomonas*. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas*. Ammonium sulphate solution (0·1 ml.) was added from the side arms after temperature equilibration to give in the suspensions the initial ammonia concentrations indicated. Duration of experiment, 120 min. Dotted curve represents the results of Meyerhof (1917) redrawn to the same maximum rate of O_2 uptake as was observed in the present experiments.

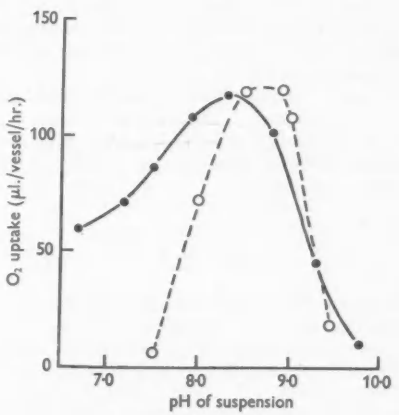

Fig. 3. The relation between pH of the suspension medium and rate of O_2 uptake by *Nitrosomonas*. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas* made up in phosphate-borate buffer of the required pH (for details of preparation see Materials and Methods section). Ammonium sulphate solution (0·1 ml.) was added from the side arm after temperature equilibration to give an initial concentration in the suspension of 10^{-2} M-$(NH_4)_2SO_4$. Duration of experiment, 120 min. Dotted curve represents the results of Meyerhof (1917) redrawn to the same maximum rate of O_2 uptake as was observed in the present experiments.

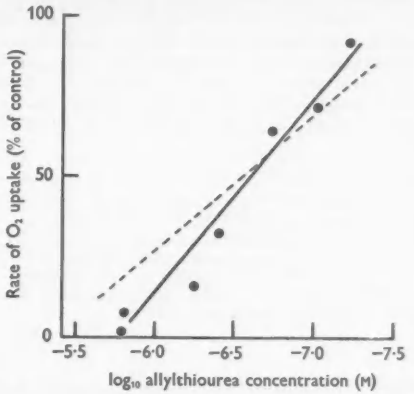

Fig. 4. The inhibition by allylthiourea of ammonia oxidation by *Nitrosomonas*. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas* made up in either water (control vessel) or in allylthiourea solutions of various strengths. Ammonium sulphate solution (0·1 ml.) was added from the side arms after temperature equilibration to give an initial concentration in the suspension of 5×10^{-3} M-$(NH_4)_2SO_4$. Observed rates of O_2 uptake (μl./vessel/hr.) were converted to percentages of the rate observed in the control vessel. Duration of experiment, 120 min. Dotted curve is the corresponding curve redrawn from Lees (1952*a*).

Table 2. *The absence of competition between allylthiourea and ammonia*

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in water or in 2×10^{-7} M-allylthiourea and placed in the main compartments of the Warburg vessels: 0·1 ml. $(NH_4)_2SO_4$ solution added from side arms after temperature equilibration to give in the suspensions the initial $(NH_4)_2SO_4$ concentrations shown in the table. Temp. 30°; duration of experiment, 120 min.)

Nitrosomonas suspended in	Final $(NH_4)_2SO_4$ concentration (M)	O_2 uptake (μl./vessel/hr.)	Inhibition due to allylthiourea (%)
Water	$1·8 \times 10^{-1}$	31	—
Allylthiourea	$1·8 \times 10^{-1}$	14	55
Water	$3·0 \times 10^{-2}$	172	—
Allylthiourea	$3·0 \times 10^{-2}$	90	48
Water	$5·0 \times 10^{-3}$	167	—
Allylthiourea	$5·0 \times 10^{-3}$	86	48
Water	$8·0 \times 10^{-4}$	111	—
Allylthiourea	$8·0 \times 10^{-4}$	51	54

The present results on the influence of pH on the respiration rate (Fig. 1) agree well with Meyerhof's results above pH 8·0. At pH's lower than 8·0, however, Meyerhof noted a far greater diminution of respiration than we observed. This discrepancy between our results and his was probably due to the fact that Meyerhof reduced the pH of his sus-

Table 3. *The effect of allylthiourea on the oxidation of ammonia and of hydroxylamine*

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in water or in 10^{-5}M-allylthiourea and placed in the main compartments of the Warburg vessels; 0·1 ml. $(NH_4)_2SO_4$ solution or $NH_2OH.HCl$ solution (neutralized with NaOH immediately before use) added from side arms after temperature equilibration to give in the suspensions initial concentration of 5×10^{-4}M-$(NH_4)_2SO_4$ or 10^{-3}M-NH_2OH, temp. 30°. There was no appreciable O_2 uptake in the absence of ammonia or hydroxylamine nor in their presence when boiled suspensions of *Nitrosomonas* were used. Duration of experiment, 120 min.)

Composition of suspension fluid	O_2 uptake (μmoles)	NO_2 formed during experiment: Calculated* (μmoles)	NO_2 formed during experiment: Observed (μmoles)
Ammonium sulphate	4·60	3·08	3·13
Hydroxylamine hydrochloride	1·83	1·83	1·84
Ammonium sulphate + allylthiourea	0·09	0·06	0·06
Hydroxylamine hydrochloride + allylthiourea	1·47	1·47	1·63

* Calculated from O_2 uptake

pensions from an initial value of 8·5 to lower values merely by adding hydrochloric acid directly to the suspension in the Warburg flask. To obtain a final pH of 7·6 he was compelled to add 0·45 ml. of 0·5N-hydrochloric acid to 1·5 ml. of *Nitrosomonas* suspension. The addition of such a relatively high concentration of acid must have caused a profound fall in pH of the suspension before the slow equilibration

Table 4. *The effect of hydrazine on the oxidation of ammonia and of hydroxylamine by* Nitrosomonas

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in water or 3×10^{-3}M-hydrazine sulphate and placed in main compartments of Warburg vessels; 0·1 ml. $(NH_4)_2SO_4$ solution, $NH_2OH.HCl$ solution, or water, added from side arms after temperature equilibration to give concentrations of (approximately) 4×10^{-4}M-$(NH_4)_2SO_4$ or 8×10^{-4}M-$NH_2OH.HCl$ in the suspensions. The hydrazine sulphate and hydroxylamine hydrochloride solutions were neutralized with NaOH immediately before use. Duration of experiment, 30 min. Initial NO_2-N$\simeq$0·3 μg./vessel. The formation of NO_2-N and $NH_2.OH$-N in the control vessels was from traces of ammonia in the washed suspensions (see Materials and Methods section).)

Nitrosomonas suspended in	μg. N per vessel found at the end of experiment as: NO_2-N	NH_2OH-N
Water	1·3	0
Ammonium sulphate	13·5	0
Hydroxylamine	9	21
Hydrazine	0·3	0·9
Hydrazine + ammonium sulphate	0·3	3·3
Hydrazine + hydroxylamine	0·3	29

of the hydrochloric acid with the alkaline earth carbonates, present in the suspension, raised the pH again to its final value of 7·6. It is therefore probable that most of the organisms were killed by the temporarily extremely acid medium before the experiment began. The present results, but not Meyerhof's, conform with the well recognized fact that in soils nitrification proceeds readily at pH's as low as 5·5. Above pH 8·5, Meyerhof's results and our own were in excellent agreement; at pH's above 8·5 the suspension medium exerts no buffering effect and final pH values were obtained as soon as the necessary trace of alkali was added. Agreement between our results and his was therefore to be expected.

A comparison between the present results and those of Meyerhof on the effect of ammonia concentration on oxygen consumption (Fig. 2) shows that Meyerhof found high ammonia concentrations somewhat more inhibitory than we did. The discrepancy is, however, not great and may well be due to a slight difference in ammonia tolerance between our strain of organisms and his.

The results given in Tables 3 and 4 are in agreement with the theory (Kluyver & Donker, 1926) that the oxidation of ammonia by *Nitrosomonas* proceeds via hydroxylamine. The steps envisaged by these workers were:

$$\underset{A}{NH_3} \rightarrow \underbrace{NH_2OH}_{B} \rightarrow HNO_2$$

We have now obtained experimental support for this theory as far as hydroxylamine is concerned. We have found that step *A* is inhibited by allylthiourea (Table 3) and that step *B* is inhibited by hydrazine (Table 4). Step *A* is also inhibited to some extent by hydrazine, perhaps indirectly by the accumulation of hydroxylamine resulting from the blockage of step *B*. The accumulation of hydroxylamine in the presence of hydrazine is shown by the results of Table 4. These results also show that in the absence of hydrazine no hydroxylamine accumulates during ammonia oxidation, thus confirming the results of Table 1.

The inhibition of step *A* by allylthiourea at very low concentrations (Fig. 4) suggests that, as previously postulated (Lees, 1946, 1948, 1952*a*), a metal enzyme is involved in ammonia oxidation and

that allylthiourea inhibition is due to chelation of the inhibitor with the essential metal. It now seems certain that the inhibition is not due to competition with ammonia, a theory adumbrated by Quastel & Scholefield (1951) since, as found previously by a different technique (Lees, 1952*a*) no competition between ammonia and allylthiourea could be detected (Table 2). Since the inhibition of nitrite formation by thiourea and allylthiourea is far more intense than the inhibition by any other of a wide range of chelating agents (Lees, 1952*a*) and since the thioureas are known to have a peculiarly high affinity for copper, there is good presumptive evidence for the theory already advanced (Lees, 1946) that the enzyme concerned in the primary ammonia oxidation by *Nitrosomonas* is a copper enzyme.

SUMMARY

1. The metabolism of cultures of *Nitrosomonas* has been investigated by the Warburg technique.

2. In contrast to results previously obtained by Meyerhof, the ammonia-oxidizing activities of *Nitrosomonas* in culture was found to be affected by change in pH in the same way as it is affected in soil.

3. Evidence is presented to show that the oxidation of ammonia to nitrite by *Nitrosomonas* proceeds via hydroxylamine. Hydrazine has been found to inhibit selectively the oxidation of hydroxylamine. In the presence of hydrazine, hydroxylamine accumulated, and was detected, during ammonia oxidation.

4. The oxidation of ammonia to hydroxylamine was completely inhibited by allylthiourea at concentrations which did not affect oxidation of the hydroxylamine.

We wish to thank Prof. W. O. Kermack, F.R.S., for his advice and encouragement throughout this work. One of us (T.H.) also wishes to acknowledge the receipt of a grant from the Agricultural Research Council.

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Return of Cholinesterase Activity in the Rat after Inhibition by Organophosphorus Compounds

1. DIETHYL *p*-NITROPHENYL PHOSPHATE (E600, PARAOXON)

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The inhibition of cholinesterase *in vitro* by organophosphorus compounds has generally been considered to be an irreversible process. However, many workers have shown that cholinesterase activity does return both *in vivo* and in isolated tissue preparations (Koelle & Gilman, 1946; Mazur & Bodansky, 1946; Grob, Lilienthal, Harvey & Jones, 1947; Dubois, Doull, Salerno & Coon, 1949; Freedman, Willis & Himwich, 1949; Hobbiger, 1951; Burgen & Hobbiger, 1951; Frawley, Hagan & Fitzhugh, 1952). Two alternative mechanisms of this return of activity have been considered. Koelle & Gilman (1946), Mazur & Bodansky (1946) and Grob *et al.* (1947) have suggested that this return is due to the resynthesis of new enzyme, and that the original inhibition is irreversible. However, the difference between the rates of return of activity *in vivo* after inhibition by various organophosphorus compounds indicate that this view cannot be of general application (Dubois *et al.* 1949; Callaway, Davies & Risley, 1952). An alternative mechanism is based on the view that during inhibition the enzyme is phosphorylated. On the basis of experiments with electric-eel cholinesterase inhibited by

tetraethyl pyrophosphate (TEPP), Wilson (1951) has suggested that the phosphorylated enzyme is hydrolysed, thus giving the original enzyme and a substituted alkyl phosphoric acid. Aldridge (1953*c*) has shown that the inhibition of true cholinesterase of erythrocytes by dimethyl *p*-nitrophenyl phosphate reverses rapidly and has shown that the properties of this reversal are consistent with a dephosphorylation process.

In this paper the behaviour of true and pseudo cholinesterase in the rat has been studied. It is shown that the inhibition of the pseudo cholinesterase of the rat by diethyl *p*-nitrophenyl phosphate (E 600) reverses rapidly *in vitro* and that the rate of return of activity *in vivo* is similar to that occurring *in vitro*. Rat-brain pseudo cholinesterase inhibited by E 600 recovers activity at a much slower rate than other rat pseudo cholinesterases. The pseudo cholinesterase of the brain has the same substrate pattern as the other pseudo cholinesterases and the same sensitivity to inhibitors. *In vivo* and also *in vitro* the true cholinesterase recovers to about 60% activity, after which the recovery process is much slower.

METHODS

The methods were those used by Aldridge (1950). Final substrate concentrations were: acetylcholine perchlorate (AcCh) 0·0138 M, acetyl-β-methylcholine bromide (MeCh), propionyl- (PrCh), *n*-butyryl- (BuCh), *iso*butyryl- (*iso*BuCh), *n*-valeryl- (ValCh) and *iso*valeryl- (*iso*ValCh) choline perchlorates, all 0·03 M, and benzoylcholine chloride (BzCh), 0·015 M. MeCh was obtained from L. Light and Co. Ltd. and BzCh from British Drug Houses Ltd. The remaining substrates were prepared in this laboratory. Corrections were made for non-enzymic hydrolysis of the substrate and for tissue suspension and erythrocyte blanks, by taking manometer readings for 30 min. before tipping in the substrate. On addition of the substrate, recovery of the inhibited cholinesterase commences, but it is too small a change to be detected and the output of CO_2 remains sensibly linear. Male albino rats of about 200 g. were used throughout. The rats were killed by coal gas and the tissues were quickly removed, washed with saline, blotted and weighed. The heart was perfused from the aorta until the effluent was free from blood. After mincing with scissors the tissues were dispersed in buffer using an all-glass homogenizer (Potter-Elvehjem type). Approximate concentrations of suspensions were: brain, 50 mg./ml.; heart, 80 mg./ml.; jejunum, 40 mg./ml.; submaxillary gland, 200 mg./ml.; diaphragm, 400 mg./ml.; spinal cord, 70 mg./ml. and female-rat serum 0·5 ml./ml. One ml. of these suspensions gave suitable outputs of CO_2 with AcCh as substrate; for tissues containing small amounts of pseudo cholinesterase (such as brain) 3 ml. of suspension were taken with BuCh as substrate.

Reversal of inhibition in vitro. The method of studying reversal *in vitro* was essentially that of Aldridge (1953*c*). Excess E 600 was removed by a preparation of rabbit serum (A-esterase) previously treated with 10^{-6} M-di*iso*propylphosphorofluoridate (DFP) to remove cholinesterase activity. After tissue suspensions had been incubated for 30 min. in bicarbonate saline (0·0312 M-$NaHCO_3$, 0·15 M-NaCl, pH 7·8) with suitable concentration of E 600 at 37°, A-esterase preparation was added to them (approx. 1 ml. to not more than 8 ml. of suspension) and they were left for a further 30 min. to destroy all inhibitor. After this 60 min. incubation with inhibitor and serum, the first determination of cholinesterase activity was made, and the inhibition was defined as 100% inhibition at zero time. At intervals suitable volumes were taken with a syringe pipette and assayed for activity. Controls without E 600 were treated similarly. In experiments lasting for 6 hr. bacterial growth was prevented by adding Merthiolate (Eli Lilly and Co. Ltd.) in 1:10 000 concentration. At this concentration Merthiolate does not interfere with cholinesterases. Suspensions of heart and, particularly, of jejunum often began to lose activity after 5 hr., but preparations of brain and spinal cord remained stable up to about 7 days. The pH remained approximately 7·8. In one experiment in which concentrated brain suspension was prepared in 0·067 M-phosphate buffer (Sørensen's), pH 7·6, and penicillin was added in addition to the Merthiolate, the true cholinesterase remained stable up till 13 days.

Inhibition in vivo. White male rats were given E 600 (0·25 mg./kg.) in saline into the tail vein or, for experiments on the intestine, by stomach tube (2·4 mg./kg.) in saline. These doses killed two to five out of every ten animals. All animals showed symptoms of salivation, fasciculations and tremors within 5 min. following intravenous injection but after an oral dose the symptoms were more delayed and less pronounced. Acute symptoms of poisoning disappear 1–2 hr. after injection. Pairs of animals were killed after 0·5 hr. and at stated times after that. Estimations were usually made at once, but occasionally samples were stored overnight at −5°. This caused no change in the cholinesterase level. Activity was measured using AcCh and BuCh as substrates and true and pseudo cholinesterase activities were calculated in terms of AcCh (see below). It proved difficult to reduce the pseudo cholinesterase activity level of brain *in vivo* by more than 50% although the activity of true cholinesterase may be as low as 5% of normal. Diaphragm cholinesterases behave similarly. This is due to the preferential inhibition of true cholinesterase by E 600. Following intravenous injection of E 600, heart pseudo cholinesterase is inhibited 25% at the most, submaxillary gland even less, while the jejunum is unaffected. If, however, E 600 is given orally the jejunum pseudo cholinesterase is almost completely inhibited.

Determination of true and pseudo cholinesterase. It was essential to have a method suitable for the determination of the low cholinesterase activity remaining in tissues *in vivo* after administration of inhibitors and also suitable for the determination of small amounts of pseudo cholinesterase in the presence of true cholinesterase and vice versa. The specific substrates, MeCh and BzCh (Mendel, Mundell & Rudney, 1943), are not hydrolysed at a high enough rate to give a method of sufficient sensitivity. The use of an inhibitor, *NN'*-di*iso*propylphosphorodiamidic fluoride (bismono*iso*propylamino fluorophosphine oxide; mipafox; Isopestox), which has a high selectivity for pseudo cholinesterase (Aldridge, 1953*a*) has enabled the cholinesterases of rat tissues to be separated. The activity remaining after incubation of a tissue suspension with 4×10^{-6} M-mipafox for 30 min. at 37° has been taken as that of true cholinesterase. Then the difference between this and the original

uninhibited activity is the activity of pseudo cholinesterase. Fig. 1 shows that at this concentration of mipafox not more than 5% of the true enzyme is inhibited while pseudo cholinesterase activity is completely removed.

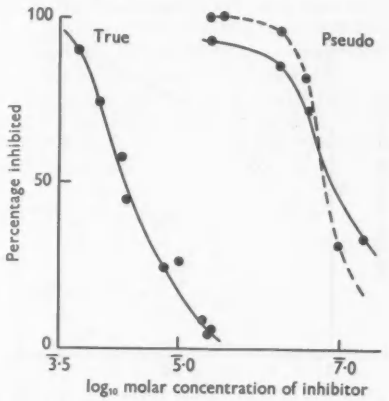

Fig. 1. Inhibition curve for rat true and pseudo cholinesterase. Brain and heart suspensions were incubated for 30 min. at 37° with mipafox and true and pseudo cholinesterase activity determined with MeCh and BuCh respectively. Broken curve, heart pseudo cholinesterase; continuous curves, brain true and pseudo cholinesterase.

The results in Table 1 show that the activity of true and pseudo cholinesterases of different tissues against AcCh, PrCh and BuCh are similar. Thus the mean activities represent the substrate hydrolysis by true and pseudo cholinesterase since there are only small differences in values for individual tissues.

The percentage of true and pseudo cholinesterase has been determined by the following method. The ratio of the activity against AcCh to that against BuCh varies from 45·5 for true cholinesterase to 0·69 for pseudo cholinesterase. Fig. 2 shows the value of this ratio for mixtures of true and pseudo cholinesterase (expressed as percentage activities in terms of activity against one substrate). From the ratio determined for an unknown tissue its composition in terms of activity against AcCh can be obtained. The activity of each

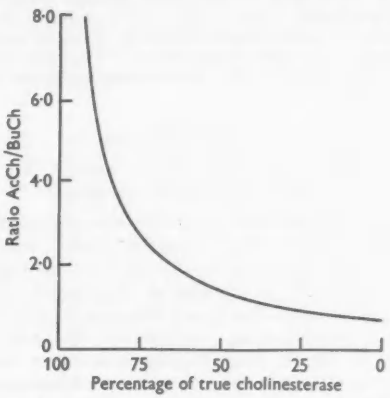

Fig. 2. Calibration curve for determining percentage of true and pseudo cholinesterase of the rat. The ratio AcCh/BuCh is determined as described in the text.

Table 1. *Relative rates of hydrolysis of substrates by various rat tissues*

(The results were obtained by the method described in the text using AcCh, 0·0138 M; BuCh and PrCh, 0·03 M and mipafox, 4×10^{-6} M. AcCh rates in each case taken as 100.)

	True cholinesterase			Pseudo cholinesterase		
	No. of exps.	BuCh	PrCh	No. of exps.	BuCh	PrCh
Brain	3	2·3	57	1	141	260
Jejunum	3	2·3	61	3	150	247
Heart	2	3·7	86	1	148	230
Submaxillary gland	2	1·5	65	1	145	191
Diaphragm	1	3·8	65	1	150	244
Serum	2	0·5	56	3	140	237
Mean relative rate of hydrolysis		2·2	65		145	245

Table 2. *Normal values for substrate hydrolysis by rat cholinesterases*

(Values have been determined using 0·0138 M-AcCh and 0·03 M-BuCh expressed as μl. CO_2/g. wet wt./hr.; the percentage of true cholinesterase (ChE) has been calculated as in the text, and corrected mean values for true (ACh) ChE and pseudo (ACh) ChE are recorded together with the standard deviation.)

Rat tissue	AcCh hydrolysis (μl. CO_2/g./hr.)	BuCh hydrolysis (μl. CO_2/g./hr.)	Percentage of true ChE as AcCh	True ChE activity as AcCh (μl. CO_2/g./hr.)	Pseudo ChE activity as AcCh (μl. CO_2/g./hr.)
Brain	10 883 ± 798	1 292 ± 115	94	10 168 ± 862	752·5 ± 86·5
Heart	5 940 ± 1 172	7 490 ± 1 110	12	856 ± 1 511	5 039 ± 514
Jejunum	12 940 ± 1 572	17 600 ± 1 625	5	751 ± 582	12 944 ± 1 352
Diaphragm	1 609 ± 343	859 ± 202	62	1 007 ± 337	602 ± 144·5
Submaxillary gland	2 865 ± 659	2 437 ± 530	42	1 210 ± 515	1 655 ± 342·5

enzyme against either substrate may be readily calculated. A similar procedure may be carried out with any pair of substrates and has been done for AcCh and PrCh. The results have always agreed well with those obtained using AcCh and BuCh. In Table 2 are the results obtained using these methods for the normal levels of true and pseudo cholinesterase for various tissues of the rat. Since all activity against choline esters is removed by 10^{-4} M-eserine, it has been assumed that only cholinesterases hydrolyse these esters.

RESULTS

Inhibition of cholinesterase in vitro *of rat by diethyl* p-*nitrophenyl phosphate*

The concentrations of diethyl *p*-nitrophenyl phosphate (E 600) were determined which produced 50% inhibition of true and pseudo cholinesterase in 30 min. at 37° in suspensions of different tissues using MeCh and BuCh as substrates for true and pseudo cholinesterase respectively. The ratios of 50% inhibition values for pseudo and true cholinesterase (Table 3) show that true cholinesterase is selectively inhibited *in vitro*. For instance in brain when 90% of the true cholinesterase was inhibited only 50–60% of the pseudo was inhibited. It is probable that the differences in 50% inhibition values for the enzyme from various tissues are due to the varying levels of enzyme hydrolysing the inhibitor in each tissue, for rat blood, which contains the highest concentration of A-esterase (Aldridge, 1953*b*), requires the highest concentrations of E 600 for inhibition.

Table 3. *Inhibition of cholinesterases of various tissues of rat by diethyl* p-*nitrophenyl phosphate*

(The molar concentrations producing 50% inhibition were determined on tissues by incubating with E 600 for 30 min. at 37° in Warburg buffer. MeCh and BuCh, 0·03 M, have been used throughout for determination of true and pseudo cholinesterase respectively. Log percentage activity was plotted against concentration of E 600 and the best straight line drawn; concentrations producing 50% inhibition were read from the curve. The inhibitor ratio is the concentration producing 50% inhibition of pseudo cholinesterase over the concentration producing 50% inhibition of true cholinesterase.)

Tissue	Molar concentration for 50% inhibition: True cholinesterase	Molar concentration for 50% inhibition: Pseudo cholinesterase	Inhibitor ratio
Blood	$1·1 \times 10^{-7}$	$2·8 \times 10^{-7}$	2·55
Brain	$1·6 \times 10^{-8}$	$3·7 \times 10^{-8}$	2·30
Heart	$7·4 \times 10^{-9}$	$2·6 \times 10^{-8}$	3·5
Jejunum	—	$1·6 \times 10^{-8}$	—

Aldridge (1950) showed that straight lines were obtained when log percentage activity of washed sheep red cells inhibited with E 600 was plotted against time. If jejunum pseudo cholinesterase is inhibited with E 600 straight lines do not result when $\log_{10}$ (percentage activity) is plotted against time (Fig. 3, broken line). Similar curves have been interpreted (Aldridge & Davison, 1952) as being due to

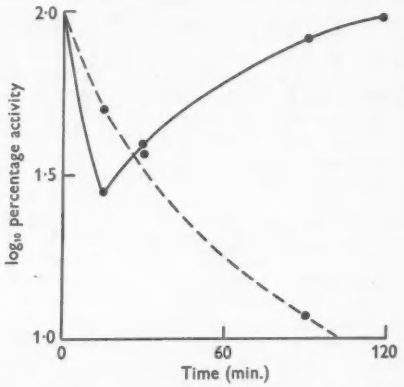

Fig. 3. Inhibition of rat-jejunum pseudo cholinesterase by E 600 (5×10^{-8} M in $NaHCO_3$, NaCl buffer). The continuous line represents the effect of adding A-esterase preparation after 15 min. on the normal progressive inhibition curve shown by the broken line.

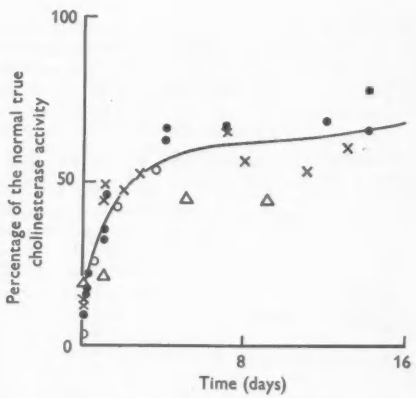

Fig. 4. Recovery of inhibited true cholinesterases of brain *in vivo* (●) and *in vitro* (×), spinal cord *in vitro* (○) and diaphragm *in vivo* (△). Each point represents the mean result for two animals for the *in vivo* experiments. Dose of E 600 was 0·25 mg./kg. by intravenous injection.

the hydrolysis of the inhibitor, but rat jejunum contains very little of the enzyme hydrolysing E 600 (Aldridge, 1953*b*). Fig. 3 also shows that, if A-esterase is added after 15 min. to jejunum treated with E 600, instead of progressive inhibition (broken curve), the inhibition is progressively reversed. This recovery is rapid. The shape of the

curve obtained when E 600 is not removed (Fig. 3) could be explained by an equilibrium being reached between the inhibitor and reversal processes. Using the reversal technique the cholinesterases of brain, spinal cord and blood have been found to recover slowly after inhibition by E 600 (Fig. 4). By 4 days, 50–60 % activity is reached; after that the recovery process is very slow. In a single experiment the recovery of inhibited true cholinesterase of rat erythrocytes was followed for 2 days. The rate of recovery was comparable to that of the true cholinesterase of brain.

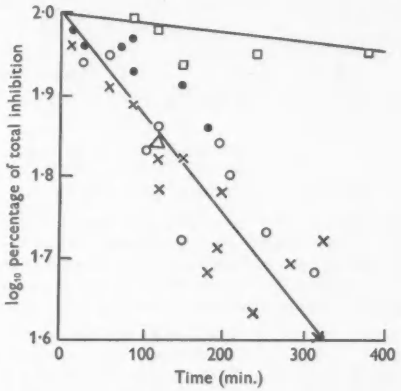

Fig. 5. Recovery of pseudo cholinesterase *in vitro* after inhibition with E 600. Total inhibition = inhibition at zero time. Pseudo cholinesterases of serum (○), jejunum (●), heart (×), diaphragm (△) and brain (□) are shown.

The pseudo cholinesterase of serum, diaphragm, submaxillary gland, heart and jejunum recovered rapidly (within about a day) and it was possible to examine the reversal directly in the Warburg flasks. The recovery process was exponential and has been treated as a first-order process and the approximate first-order constants and half-life periods for recovery determined (Table 4). By contrast, the recovery rates of pseudo cholinesterase of brain and spinal cord were slow. The recovery curve appeared to be exponential but as the pseudo cholinesterase was unstable it was impossible to study the recovery for more than 7 days (63 % reversal). In Fig. 5 all the results for the recovery of pseudo cholinesterases after inhibition with E 600 are recorded. The wide scatter of points is probably due to interaction of A-esterase preparation and the suspension since controls to which A-esterase preparation have been added also show a large variation under similar conditions. Inhibited jejunum pseudo cholinesterase appears to recover slightly more slowly than the pseudo cholinesterase of heart and serum, but this is probably due to destruction of the recovered cholinesterase, for jejunum pseudo cholinesterase is the least stable of those examined. It may be said, however, that the rates of recovery of inhibited pseudo cholinesterase *in vitro* for heart, serum, submaxillary gland, diaphragm and jejunum are similar. The striking difference between rate of recovery of brain pseudo cholinesterase and that of other tissues is also seen in Fig. 5.

Table 4. *Constants for rate of reversal of diethyl-p-nitrophenyl-phosphate-inhibited pseudo cholinesterase* in vivo *and* in vitro

(The pseudo cholinesterase activity was determined with BuCh (0·03 M). *In vitro* and *in vivo* experiments were as described in text. Half-life periods are the mean times required for 50 % recovery of the inhibited pseudo cholinesterase, determined directly from the recovery curves. First-order constants were the mean values for the constant determined from the slope of the best straight line, calculated by the method of least squares, when log percentage total inhibition was plotted against time.)

Pseudo cholinesterase	Half-life periods for recovery: *In vivo* (hr.)	Half-life periods for recovery: *In vitro* (hr.)	First-order constant *in vitro* K (min.$^{-1}$)
Brain	96	103	1×10^{-4}
Heart, jejunum, serum and submaxillary gland	4·9	5	2×10^{-3}

When the pseudo cholinesterases in horse and human plasma were inhibited with E 600, the reversal technique showed that the human-plasma enzyme recovered much more slowly than the horse-plasma enzyme. Horse plasma showed a slight recovery of activity in 5 days while no change could be detected in human plasma. Furthermore, no difference in curves of inhibition by E 600 of horse plasma was detected whether A-esterase was added after 15 min. or not. Hobbiger (1951) has shown that guinea-pig serum inhibited with TEPP recovers from 8·5 % of its original activity at zero time to 55 % in 72 hr. but that human serum similarly inhibited shows no recovery in the same period.

Inhibition of rat cholinesterase by diethyl p-nitrophenyl phosphate in vivo

It was found that the pseudo cholinesterases of diaphragm, heart and jejunum rapidly recovered their activity *in vivo*, whereas true cholinesterases of brain and diaphragm, and pseudo cholinesterase of brain took much longer to do so.

It was not possible completely to inhibit all the pseudo cholinesterases *in vivo* (for example, heart and brain) without killing the animal. Therefore, on the basis of an exponential rate and to facilitate comparison, the activity of the tissue half an hour after the administration of E 600 has been taken as

Table 5. *Relative rates of hydrolysis of substrates by rat-brain and heart pseudo cholinesterases*

(Suspensions were incubated without and with 4×10^{-6} M-mipafox for 30 min. at 37°. The substrates were added immediately and the activities determined. The ratios of substrate activities inhibited by the mipafox were determined. AcCh values were taken as 100 in each case and the corresponding values for other substrates calculated.)

	BuCh	*iso*BuCh	PrCh	ValCh	*iso*ValCh	BzCh
Rat brain	141	150	260	90	73	37
Rat heart	148	151	230	82	75	30

representing 100% inhibition and the percentage increase in activity (loss of inhibition) has been plotted against time (Fig. 6). As may be expected, there was a large scatter of results. The half-life values for reversal were calculated as previously (Table 4). Brain pseudo cholinesterase was found to recover much more slowly (half-life period about 96 hr.) than other pseudo cholinesterases (half-life period about 4·9 hr.). Results for true cholinesterase of brain, spinal cord and diaphragm (Fig. 4) show that recovery from inhibition with E 600 takes place slowly until on the fourth day, when 50–60% of normal brain cholinesterase activity has returned, reversal slows down considerably.

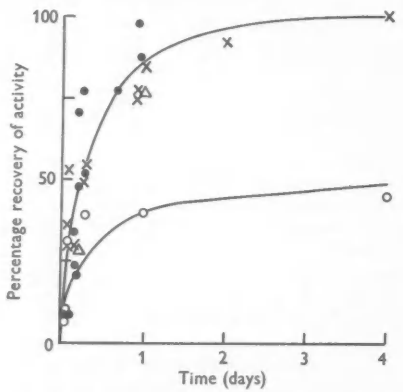

Fig. 6. Recovery of pseudo cholinesterases of the rat *in vivo*. Rats were given 0·25 mg./kg. E 600 intravenously or for the jejunum experiments 2·4 mg./kg. orally. Each point represents the pseudo cholinesterase activity of the tissue determined as described in the text. Zero activity = the activity after 30 min.; 100% activity = the normal activity of the tissue. Brain (○), heart (×), jejunum (●) and diaphragm (△).

Figs. 5 and 6 show that after inhibition by E 600 *in vitro* and *in vivo* the recoveries of heart, jejunum, diaphragm, serum and submaxillary gland pseudo cholinesterases are similar.

Pseudo cholinesterase of brain

Since a difference between rates of recovery of the pseudo cholinesterase of brain and that of other tissues had been demonstrated, it seemed important to see if any difference between the two enzymes could be detected by more conventional methods. Accordingly, hydrolysis rates against seven substrates were determined for the pseudo cholinesterase of brain and heart (Table 5). Inhibition values for the pseudo cholinesterase of brain and heart using BuCh as substrate were also determined for four different inhibitors (Table 6). In all respects the behaviour of the pseudo cholinesterases was similar.

Table 6. *Comparison of inhibitor values for pseudo cholinesterase of brain and heart*

(The inhibitor was incubated with brain and heart suspension at 37° for 30 min. before adding the BuCh. Log percentage activity was plotted against concentration and the best straight line drawn. Concentrations producing 50% inhibition were read from the curve.)

	Molar concentration for 50% inhibition	
Inhibitor	Brain	Heart
DFP	$2·6 \times 10^{-8}$	$1·6 \times 10^{-8}$
DPDA*	$5·2 \times 10^{-7}$	$4·6 \times 10^{-7}$
Mipafox	$1·7 \times 10^{-7}$	$1·7 \times 10^{-7}$
E 600	$3·7 \times 10^{-8}$	$2·6 \times 10^{-8}$

* Di*iso*propylphosphorodiamidic anhydride.

Table 7. *Loss of cholinesterase activity on keeping suspensions at 37°*

Suspensions of rat brain were prepared in $NaHCO_3$, NaCl buffer without a preservative. True and pseudo cholinesterase values, using 0·03 M MeCh and BuCh respectively, were measured on 1 ml. of suspension from the same tube. Each result is the mean of two observations.)

Time (days)	True cholinesterase (μl. CO_2/ml./min.)	Pseudo cholinesterase (μl. CO_2/ml./min.)
0	12·26	3·45
4	2·81	3·11
5	2·21	2·27
6	1·87	2·70

The recovery rate of brain pseudo cholinesterase, after inhibition with E 600, is closer to the recovery rate of true cholinesterase of brain than it is to that of the pseudo cholinesterase of other tissues. It is important therefore to show clearly that two discrete cholinesterases are in fact present. On keeping a rat-brain suspension at 37° without a preservative

for a number of days, the true cholinesterase of the suspension decreased quickly while the pseudo cholinesterase did so only slowly (Table 7). Indeed it was possible to destroy almost all the true cholinesterase before most of the pseudo cholinesterase had gone. In the presence of Merthiolate under similar conditions pseudo cholinesterase is the least stable. This must indicate that two enzymes of different stability are present. It will also be seen that the substrate patterns in the presence and absence of 4×10^{-6}M-mipafox (Table 1) indicate a very distinct difference in behaviour of the true and pseudo cholinesterase of brain and that in this respect they resemble the true and pseudo cholinesterases of all the rat tissues so far examined. This confirms the findings of Ord & Thompson (1950) who showed both true and pseudo cholinesterase were present in the rat brain. The two enzymes have also been identified in both human (Ord & Thompson, 1952) and dog brain (Burgen & Chipman, 1951).

DISCUSSION

The recovery rates of rat cholinesterases after inhibition by E 600 have been studied. It has been suggested that recovery from cholinesterase inhibition was due to resynthesis of new enzyme. However, varying rates of recovery after inhibition by organophosphorus inhibitors have been reported for man (Grob, Garlick & Harvey, 1950; Callaway *et al.* 1952), suggesting that regeneration of the enzyme is certainly not the sole factor concerned in the reversal of inhibition. Similarly, rat-serum cholinesterase (largely pseudo cholinesterase) has been found to recover in about 10 days after inhibition by DFP (Koelle & Gilman, 1946), whereas recovery after inhibition by E 600 has been shown in this paper to be much more rapid. Sprinson & Rittenberg (1949) have shown that the half-life period of rat-serum protein synthesis is about 7 days. It is therefore unlikely that resynthesis of the enzyme can play a significant part in the rapid recovery of pseudo cholinesterase after inhibition by E 600. This view is confirmed by the fact that the recovery rates *in vivo* are similar to rates *in vitro* when resynthesis presumably cannot occur.

Wilson (1951), Burgen & Hobbiger (1951) and Aldridge (1953*c*) have suggested that the enzyme is phosphorylated by the organophosphorus inhibitors and that reversal is in fact the hydrolysis of the enzyme phosphate. It may be expected that this phosphorylated enzyme would have a definite hydrolysis rate differing from one substituted phosphate (e.g. dimethyl, diethyl or di*iso*propyl) to another (Burgen & Hobbiger, 1951) and from one enzyme to another. Hydrolysis of the phosphorylated enzyme would then be expected to be an exponential process. This has been found for pseudo cholinesterase reversal only. If the substituted phosphate is kept the same, in this case diethyl phosphate, different phosphorylated enzymes would be expected to have different hydrolysis rates. For example, reversal of human-plasma pseudo cholinesterase inhibited with E 600 was not detected in 5 days while recovery of horse-plasma pseudo cholinesterase was very slow when compared to that of rat plasma. Similarly, Hobbiger (1951) noted a difference between the rate of reversal of inhibition with TEPP of guinea pig and human-plasma pseudo cholinesterases. A difference between horse and human pseudo cholinesterase has been demonstrated by Kalow (1952) in spectrophotometric studies with procaine as substrate and also by Lévy & Denys (1951) with various substrates. It is, therefore, suggested that the reversal constants for inhibited enzymes may be a useful criterion of an enzyme's identity.

There is a very striking difference between the rates of recovery, following inhibition by E 600 of the pseudo cholinesterase of brain and spinal cord on the one hand and other pseudo cholinesterases on the other. In the central nervous system the pseudo cholinesterases recover at a similar rate to the true cholinesterase. Yet substrate patterns and inhibitory values have revealed no differences between the pseudo cholinesterase of heart and brain while the difference between the relative stabilities of the true and pseudo cholinesterase of brain, together with the different substrate patterns of the two enzymes (Table 1), emphasizes that there are two discrete cholinesterases in the brain. Thus, the reversal rates have shown a distinction between the pseudo cholinesterase of the central nervous system and that of other tissues which was undetected by conventional methods.

The brain differs from other tissues in having a high lipid content and it may be argued that this could act as reservoir of dissolved E 600 and thus prevent the cholinesterase from returning to normal at the usual rate. However, as A-esterase is present at least in the *in vitro* preparation, it is likely that any E 600 slowly liberated would be hydrolysed.* Different amounts of free amines or other compounds in the tissues might possibly exert a catalytic effect on the return of cholinesterase activity, but not in the brain. It seems more likely that the difference in reversal rates is due to a difference in stability of the phosphorylated enzyme, and that this, in turn, indicates some difference between brain and tissue pseudo cholinesterases, e.g. brain cholinesterase being a lipoprotein (Ord & Thompson, 1951).

* *Note added in proof.* Without Merthiolate and penicillin the rate of return *in vitro* of brain pseudo cholinesterase is the same as for other tissue preparations.

The true cholinesterase of the nervous system of the rat has been shown to recover quickly at first, thereafter only slowly. It is particularly interesting that the true cholinesterase of brain shows a similar recovery pattern *in vivo* and *in vitro*, for it would be expected that recovery would be exponential. There seem to be two possible explanations of the findings. It may be that part of the inhibited enzyme (about 40%) is removed as a foreign protein, and that all the remaining inhibited enzyme has recovered its activity after 4 days. The further slow rise in activity would then be due to enzyme resynthesis. Since the recovery rate is the same *in vitro* one must assume that the inhibited enzyme is also removed *in vitro*. A second possibility is that two enzymes are present in the true cholinesterase, one (60% of the whole) which recovers rapidly and a second enzyme whose rate of recovery is slow. In view of the demonstration of the difference between the pseudo cholinesterase of brain and spinal cord and that of other tissues this must be considered.

SUMMARY

1. After inhibition of rat pseudo cholinesterase of heart, serum, jejunum and diaphragm by diethyl *p*-nitrophenyl phosphate (E 600) the total recovery of enzyme activity occurs in about a day both *in vivo* and *in vitro*.

2. A difference in recovery of activity (*in vitro* and *in vivo*) between the pseudo cholinesterase of brain and the other pseudo cholinesterases examined has been shown.

3. The recovery of true cholinesterase after inhibition with E 600 occurs slowly (60% in 4 days) both *in vivo* and *in vitro*.

My thanks are due to Dr W. N. Aldridge for advice and to Miss J. I. Wheatley for technical assistance.

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Studies of Cholesterol Biosynthesis

1. A NEW CHEMICAL DEGRADATION OF CHOLESTEROL

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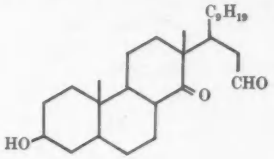
The study of cholesterol biosynthesis from precursors labelled with isotopic carbon would be greatly assisted if methods were available for isolating each of the twenty-seven carbon atoms of the sterol in a form suitable for isotopic assay. The constituent atoms in the side chain can be separated (Wüersch, Huang & Bloch, 1952) by adapting known procedures originally designed to determine the structure of cholesterol, but known degradations of the alicyclic ring structure do not easily lend themselves to the separation of individual atoms. New procedures were therefore devised; the first of these, aimed at rings *A* and *B*, has been worked out in detail.

Our point of departure was the observation of Achtermann (1934) and of Laucht (1935) that the keto aldehyde (I), from ergosterol, on pyrolysis gives the ketone (II) and the $\alpha\beta$-unsaturated

aldehyde (III). The process may be viewed as the reversal of a Michael addition reaction (II + III = I).

This suggested that if ring *B* of cholesterol were opened by oxidation, further manipulation might afford the keto aldehyde (IV), which on pyrolysis should give ring *A* as 2-methyl*cyclo*hexanone (V). Separation of the seven carbon atoms in 2-methyl-*cyclo*hexanone could then be undertaken.



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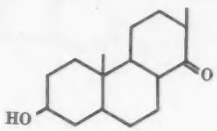

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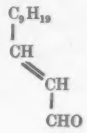

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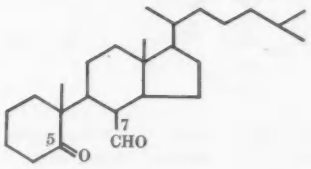

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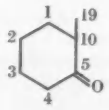


V

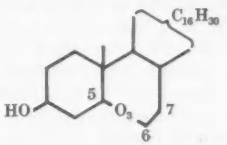
A smooth dehydration of the aldehyde to the Δ^3-unsaturated ketone could not be effected. Ethanolic hydrogen chloride gave a complex mixture, and ethanolic sulphuric acid led by a double dehydration to 6-formyl-*B*-norcholesta-3:5-diene (VIII), isolated by chromatography as a viscous yellow oil. The structure (VIII) is indicated by the ultraviolet light absorption of the substance ($\lambda_{max.} = 293\,m\mu.$; $\epsilon = 18\,150$) and of its 2:4-dinitrophenylhydrazone ($\lambda_{max.} = 402\,m\mu.$; $\epsilon > 35\,500$). The ultraviolet light absorption data obtained throughout this work conformed closely with the generalizations of Woodward (1941, 1942) and of Evans & Gillam (1941, 1943, 1945) for unsaturated carbonyl compounds, and with those of Roberts & Green (1946) for the 2:4-dinitrophenylhydrazones.

The steroid nomenclature used throughout this paper follows the recommendations given in the report of the 1951 Ciba Foundation Conference (*J. chem. Soc.* 1951, p. 3526). It is to be noted that, after removal of C-6, the original C-7 becomes C-6 in the names of the *B*-nor derivatives.

METHODS AND DISCUSSION

The ozonolysis of cholesterol was first studied. Cholesterol ozonide (VI) was originally made by Harries (1912), and optimum conditions for its formation were examined by Berenstein, Georg & Briner (1946). The latter authors attempted, with small success, to reduce the ozonide; however, they did not try the action of zinc dust in cold acetic acid, which we find to give an almost quantitative yield of 5:6-*seco*cholestan-6-al-3-ol-5-one (VII; R = OH). This crystallized with a firmly bound molecule of ether; it was characterized as a 3:5-dinitrobenzoate and as a 2:4-dinitrophenylhydrazone. The aldehyde

On treatment with sodium ethoxide in ethanol, the *seco*aldehyde (VII; R=OH) passed smoothly into 6-formyl-*B*-norcholest-5-en-3-ol (IX; R=OH). This substance formed a hydrate from which water could not be expelled without loss of crystalline form, but the 2:4-dinitrophenylhydrazone was anhydrous. The noraldehyde (IX; R=OH) was a sensitive substance: on exposure of a hexane solution to air two new products, one neutral and one acidic, were formed. Since these readily liberated iodine from potassium iodide in acetic acid, they are



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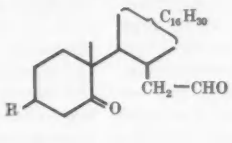

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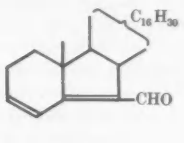
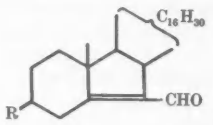


VIII

formulated tentatively as 6-formyl- and 6-carboxy-5:6-epoxy-*B*-norcholestan-3-ol (X; R=CHO and CO_2H) respectively. Attempts to oxidize the noraldehyde (IX; R=OH) to the corresponding carboxylic acid by means of permanganate or silver oxide (Pearl, 1946*a*, *b*) gave unsatisfactory products.

Preliminary attempts were now made to split the diketo acid (XII; R=OH) to give 2-methyl*cyclo*hexenone or 2-methyl*cyclo*hexanolone. Even in a drastic pyrolytic experiment, however, only a mixture of *iso*octane and *iso*octene was isolated. It was accordingly decided to replace the 3-hydroxyl



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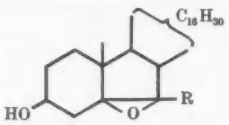
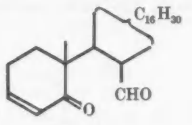


X



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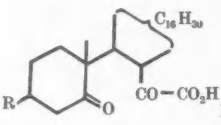
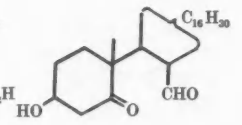
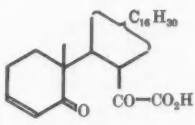


XII

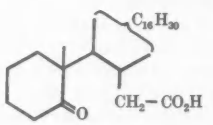


XIII

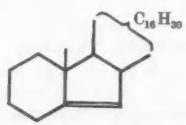


XIV

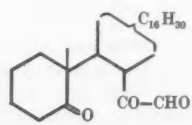
Ozonization of the noraldehyde (IX; R=OH), followed by reduction with zinc and acetic acid, afforded a crystalline acidic product along with a much smaller amount of neutral material. From the latter, a very small amount of a 2:4-dinitrophenylhydrazone, probably that of 5:6-*seco*-*B*-norcholest-3-en-6-al-5-one (XI) was obtained. The acid proved to be 5:6-*seco*cholest-3-ol-5:7-dion-6-oic acid (XII; R=OH). It lost carbon dioxide readily, not only on heating with aniline, a reaction typical of α-keto acids, but also on attempted preparation group by a hydrogen atom before carrying out further experiments. The required 5:6-*seco*cholestan-5:7-dion-6-oic acid (XII; R=H) was soon obtained from the unsaturated diketo acid (XIV) by catalytic reduction with palladized charcoal and hydrogen. The over-all yield was very moderate, and it was found that the same compound was more easily obtainable and in higher yield by carrying out a variation of the series of reactions described above, involving the substitution of cholest-5-ene for cholesterol.



XV



XVI



XVII

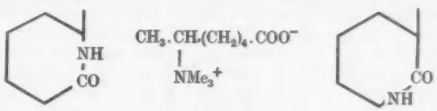
of the 3:5-dinitrobenzoate and 2:4-dinitrophenylhydrazone, the derivatives actually isolated being those of the decarboxylated product, 5:7-*seco*-*B*-norcholestan-6-al-3-ol-5-one (XIII). No rearrangement had occurred in the formation of the diketo acid (XII; R=OH), for, by dehydration with ethereal sulphuric acid, it was smoothly convertible into the corresponding unsaturated diketo acid (XIV). This compound had the characteristic ultraviolet light absorption of an αβ-unsaturated ketone. If a shift of the double bond in IX (R=OH) had led to the formation of a compound with the α-keto acid side chain attached at carbon 5, no such dehydration product would have been obtainable.

Cholesterol was converted into cholesteryl chloride by a method similar to that used by Diels & Blumberg (1911). Reduction with sodium and *n*-amyl alcohol (Mauthner & Suida, 1894) then led to the production of cholest-5-ene in 77% over-all yield. Ozonization followed by reduction with zinc and acetic acid afforded 5:6-*seco*cholestan-6-al-5-one (VII; R=H) as a colourless oil which formed a crystalline 2:4-dinitrophenylhydrazone. Fission of the ozonide with aqueous hydrogen peroxide gave the corresponding acid (XV; Lettré, 1933) which was isolated as a crystalline hydrate. Neither of the reported methods (Lettré, 1933) for the conversion of this acid into *B*-norcholest-5-ene (XVI), a possibly

useful intermediate for our purposes, was found to be satisfactory owing to the low yields and difficulties in crystallization. However, the *seco*aldehyde (VII; R=H) was converted easily into the *B*-noraldehyde (IX; R=H), a viscous lemon-yellow oil characterized as its red 2:4-dinitrophenylhydrazone. Ozonization, reduction, and separation of the acidic fraction then gave the desired diketo acid (XII; R=H), identical with the product obtained previously; but in this case an approximately equivalent amount of material was present in the neutral fraction as a viscous pale-yellow oil. Chromatography on alumina (British Drug Houses Ltd., chromatographic grade), washed with methyl formate, separated this oil into two main fractions. (We are indebted to Dr P. A. Robins of this Institute for this unpublished method of treating alumina.) The one which was less strongly adsorbed formed colourless crystals from ethanol, and was apparently 5:6-*seco*cholestan-6-al-5:7-dione (XVII). The other (smaller) fraction failed to crystallize and analytical figures indicated a formula $C_{27}H_{46}O_4$. It was not further investigated.

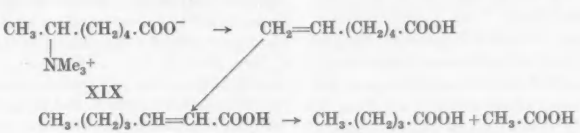
When the 5:6-*seco*cholestan-5:7-dion-6-oic acid (XII; R=H) was heated with aniline, carbon dioxide was slowly evolved, although we were unable to isolate any product of decarboxylation from the reaction. However, a similar reaction was found to take place when the free diketo acid was heated alone above 200°, carbon dioxide being slowly formed (cf. Darzens & Levy, 1937). In this case, chromatography of the residual neutral fraction from the pyrolysis led to the isolation, in relatively poor yield, of the crystalline *seco*noraldehyde (IV). Thus, our primary objective had been attained. Pyrolysis of this aldehyde with various catalysts did indeed lead to the production of 2-methyl*cyclo*hexanone (V), the best yield being obtained in the presence of potassium carbonate at 450–500°. The identity of the ketonic product was established by conversion to its 2:4-dinitrophenylhydrazone, the melting point of which was undepressed when mixed with the authentic 2-methyl*cyclo*hexanone derivative. Owing to the loss involved in the isolation of the crystalline aldehyde (IV), it was found best to use directly the crude product obtained after pyrolysis of the diketo acid. At the same time, the diketoaldehyde (XVII) was found to yield 2-methyl*cyclo*hexanone under similar conditions; again, the crude product was used for routine work. Although the amount of 2-methyl*cyclo*hexanone isolated was small (yield about 10%), it was sufficient for our purposes as all the other stages proceeded in excellent yield.

All that was now required was a stepwise degradation of 2-methyl*cyclo*hexanone. This ketone readily underwent the Schmidt reaction in aqueous hydrochloric acid solution. The crude ϵ-amino-*n*-heptanoic lactam (XVIII), obtained in almost quantitative yield, had m.p. 70–75°, which rose to 91° after three recrystallizations from light petroleum. Since the loss involved in purification by this method was over 50%, further work was



XVIII XIX XX

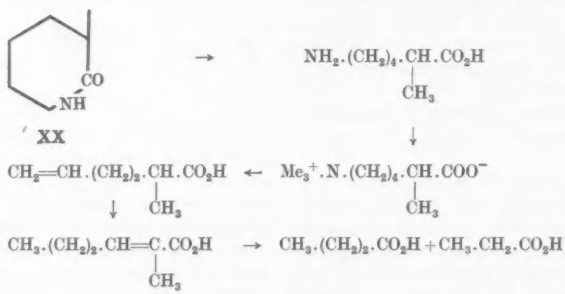
carried out on the crude product. Acid hydrolysis (Eck, 1937) gave ϵ-amino-*n*-heptanoic acid hydrochloride (Ungnade & McLaren, 1945) as a colourless gum. The pure material could readily be isolated by precipitation from ethanolic solution with ether. For our purposes, the gummy material was sufficiently pure for further experiments. Methylation with methyl iodide in methanol in the presence of silver oxide gave *n*-heptanoic ϵ-trimethylbetaine (XIX) in good yield. Although the free betaine proved to be a highly deliquescent substance, the beautifully crystalline hydrochloride could be purified. The method seems to be superior to the one involving methyl sulphate used by Giral (1935) for the preparation of *n*-hexanoic ϵ-trimethylbetaine. Fusion of the crude betaine (XIX) at 350° with potassium hydroxide gave, as expected, *n*-valeric and acetic acids in equivalent quantities, the yield of each being about 44%. This complex reaction involves: (i) elimination of trimethylamine, which occurs freely between 150 and 250°, (ii) migration of the double bond initially formed to the $\alpha\beta$-position, and (iii) fission of the $\alpha\beta$ double bond (Varrentrap, 1840; Edmed, 1898; Hunter & Popják, 1951).



In preliminary experiments, the acids were identified by chromatographic techniques (Brown, 1950; James & Martin, 1952).

The product proved to be more complex than expected, for, in addition to the *n*-valeric and acetic

acids, about 6% each of *n*-butyric and propionic acids were also detected. However, when the same degradative sequence of reactions was carried out on pure ϵ-amino-*n*-heptanoic lactam, only *n*-valeric and acetic acids appeared in significant quantity in the final acid mixture. On the other hand, material recovered from the mother liquors of the recrystallized lactam gave a very high proportion of *n*-butyric and propionic acids. It was evident that the original lactam preparation had contained also 10–15% of the isomeric ϵ-amino-α-methyl-*n*-hexanoic lactam (XX), from which the *n*-butyric acid (C-4, 3, 2 and 1 of cholesterol) and propionic acid (C-19, 10 and 5 of cholesterol) were ultimately derived.



Attempts to separate the two lactams chromatographically were only partially successful. The main component (XVIII) could be obtained pure, recovery approx. 70%, but the remaining fractions all contained a mixture of the two lactams. Purification could be carried out most efficiently by chromatography of the four acids obtained after the degradation of the mixed lactams. The chromatographic techniques are described in detail in another paper (Cornforth, Hunter & Popják, 1953).

According to Hildebrand & Bogert (1936), Beckmann rearrangement of 2-methyl*cyclo*hexanone oxime gives ϵ-amino-*n*-heptanoic lactam in 97% yield. However, their proof of the structure of the product was based on recrystallized material, and, in fact, we found that the crude product from the Beckmann rearrangement had a composition (cf. Rogovin, Khaït, Knunyants & Rymashevskaya, 1947) identical with that of the lactam obtained by the Schmidt reaction. The latter reaction is to be preferred both by virtue of the better and more reproducible yield and also because of the simpler experimental technique required.

The remainder of the degradation, i.e. the degradation of the *n*-carboxylic acids obtained from 2-methyl*cyclo*hexanone, was carried out by standard methods or slight modifications thereof and are considered in detail in the following paper in which the application of the whole process of degradation to [^{14}C]cholesterol is described (Cornforth *et al.* 1953).

EXPERIMENTAL

Products derived from cholesterol ozonide

All melting points are uncorrected.

The elaborate apparatus advocated for the preparation of cholesterol ozonide (Berenstein *et al.* 1946) can be greatly simplified. Yields of 80% were reproducibly obtained by passing excess ozone through a saturated solution of cholesterol in dry *n*-hexane until precipitation appeared to be complete. After centrifugation and washing of the product with light petroleum (b.p. 40–60°), it was dried *in vacuo* at 25° and the glassy ozonide used as such for further work. Cholesterol ozonide (7·0 g.) was heated with stirring on the steam bath with water (30 ml.) and a few ml. of 'Perhydrol', more being added as the reaction proceeded. The solid was gradually replaced by a white glue. After cooling and extraction into ether, acidic material was isolated by extraction into 2N-NaOH. Acidification, re-extraction into ether, drying, and finally evaporation to dryness *in vacuo* at room temperature gave crystalline 5:6-seco*cholestan*-3-*al*-5-*on*-6-*oic acid monohydrate* (5·5 g.), m.p. 75–80°. (Found: C, 72·1; H, 10·6. $C_{27}H_{48}O_5$ requires C, 71·7; H, 10·6%.)

On the other hand, reduction of the ozonide (15·0 g.) yielded the corresponding aldehyde. After shaking at 25° with Zn powder (15 g.) and acetic acid (100 ml.) for 60 hr., I_2 was no longer rapidly liberated from a crystal of KI added to a sample of the solution. Inorganic material was removed by filtration and washed with ether (250 ml.), and the combined filtrates were washed successively with water, 5% aqueous $NaHCO_3$ and water. Evaporation to dryness *in vacuo* at 25° left silvery leaflets of the *etherate* of 5:6-seco-*cholestan*-6-*al*-3-*ol*-5-*one* (13·1 g.), m.p. 55–60°. (Found: C, 76·0, 75·3; H, 11·1, 11·0. $C_{31}H_{56}O_4$ requires C, 75·6; H, 11·4%.) There was no significant absorption in ethanol at $\lambda > 220$ mμ. The aldehyde (0·5 g.) was allowed to stand for 20 hr. in dry benzene (15 ml.) with dry pyridine (2 ml.) and 3:5-dinitrobenzoyl chloride (1·0 g.). The crude product (0·65 g.) was obtained in the usual way, and after crystallization from ethyl acetate-aqueous methanol, it formed white microprisms of the 3:5-*dinitrobenzoate*, m.p. 88–90°. (Found: N, 5·0. $C_{34}H_{48}O_8N_2$ requires N, 4·6%.) On heating the *seco*aldehyde (0·15 g.) with a slight excess of 2:4-dinitrophenylhydrazine in ethanol (20 ml.) with subsequent

addition of a few drops of conc. HCl at the boiling point and cooling to 0°, an orange precipitate appeared. Filtration and crystallization from aqueous ethanol gave 5:6-seco*cholestan*-6-*al*-3-*ol*-5-*one* 2:4-*dinitrophenylhydrazone*, m.p. 196° decomp. (Found: C, 66·2; H, 8·3; N, 9·3. $C_{33}H_{50}O_6N_4$ requires C, 66·2; H, 8·4; N, 9·4%.) $\lambda_{max.} = 363$ mμ.; $\epsilon_{max.} = 18300$ in ethanol.

5:6-*seco*Cholestan-6-al-3-ol-5-one (5·0 g.) in dry ether (50 ml.) was allowed to stand for 24 hr. at room temperature with 10% (v/v) ethereal H_2SO_4 (50 ml.). The solution was poured into water and the ether layer separated. After washing and drying, the solvent was evaporated, leaving a yellow gum which was dissolved in a little benzene. Chromatography on Al_2O_3 (British Drug Houses Ltd., chromatographic grade) gave in the first benzene eluate 6-*formyl*-B-*norcholesta*-3:5-*diene* (3·2 g.) as a viscous yellow oil. (Found: C, 84·5; H, 10·8. $C_{27}H_{42}O$ requires C, 84·8; H, 11·0%.) $\lambda_{max.} = 293$ mμ.; $\epsilon = 18150$ in ethanol. A bright red 2:4-*dinitrophenylhydrazone*, m.p. 267° decomp., was readily obtainable and crystallized from ethyl acetate in felted needles. (Found: C, 69·9; H, 8·1; N, 9·9. $C_{33}H_{46}O_4N_4$ requires C, 70·4; H, 8·2; N, 10·0%.) $\lambda_{max.} = 402$ mμ.; $\epsilon > 35500$ in ethanol.

The same *seco*aldehyde (VII; R=OH; 13·1 g.) in ethanol (175 ml.) was allowed to stand overnight with 4% ethanolic sodium ethoxide (25 ml.). After the solution was poured into a large volume of dilute acetic acid and was extracted into ether, the product was isolated by washing, drying, and evaporation to dryness *in vacuo* at 25°. 6-*Formyl*-B-*norcholest*-5-*en*-3-*ol* (10·5 g.) formed yellow leaflets of the *monohydrate*, m.p. 60–65°. (Found: C, 77·4; H, 11·0. $C_{27}H_{46}O_3$ requires C, 77·5; H, 11·0%.) $\lambda_{max.} = 251$ mμ.; $\epsilon = 12275$ in ethanol. The corresponding 2:4-*dinitrophenylhydrazone* separated from ethyl acetate in clusters of orange needles, m.p. 248–249° decomp. (Found: C, 68·4; H, 8·3; N, 9·8. $C_{33}H_{48}O_5N_4$ requires C, 68·3; H, 8·3; N, 9·7%.) $\lambda_{max.} = 390$ mμ.; $\epsilon = 21700$ in ethanol.

When the noraldehyde (1·8 g.) was allowed to stand in *n*-hexane (25 ml.) for 7 days, a crystalline deposit (0·2 g.) appeared. Recrystallization from $CHCl_3$: *n*-hexane produced spherical clusters of fine needles, m.p. 224°, of (?) 5:6-*epoxy*-B-*norcholestan*-3-*ol*-6-*carboxylic acid hemihydrate*. (Found: C, 72·9; H, 10·2. $C_{27}H_{44}O_4 . \frac{1}{2}H_2O$ requires C, 73·5; H, 10·2%.) Evaporation of the *n*-hexane filtrate and washings gave a yellow crystalline solid (1·5 g.). Chromatography on Al_2O_3 in acetone removed impurities, and the main product was then eluted with $CHCl_3$:ethanol (1:1). Crystallization from ethanol gave (?) 5:6-*epoxy*-6-*formyl*-B-*norcholestan*-3-*ol hemihydrate* as colourless prisms, m.p. 160–165°. (Found: C, 76·1; H, 10·5. $C_{27}H_{44}O_3 . \frac{1}{2}H_2O$ requires C, 76·2; H, 10·6%.) The formyl-*B*-norcholestenol could also be dehydrated with ethereal H_2SO_4 to give the doubly unsaturated noraldehyde (VIII), previously obtained in like manner from the *seco*aldehyde (VII; R=OH).

6-Formyl-*B*-norcholest-5-en-3-ol (10·2 g.) was dissolved in dry *n*-hexane (200 ml.) and ozonized until the solution became colourless. The residue, after evaporation nearly to dryness *in vacuo*, was shaken overnight with Zn (11·0 g.) and acetic acid (100 ml.). After filtration and addition of water (200 ml.) and ether (250 ml.) to the filtrate, the ethereal extract was washed successively with water, 5% aqueous $NaHCO_3$ and water. The acidic material was then extracted into 2N-NaOH. The neutral ethereal extract, after washing, drying and evaporation *in vacuo*, left a gummy residue (about 1·0 g.) which did not crystallize. Treatment with ethanolic 2:4-dinitrophenylhydrazine in the presence of HCl led to the formation of a small quantity of 5:6-seco-B-*norcholest*-3-*en*-6-*al*-5-*one* 2:4-*dinitrophenylhydrazone* in masses of small orange prisms, m.p. 118° decomp. (Found: C, 67·7; H, 8·6; N, 10·3. $C_{32}H_{46}O_5N_4$ requires C, 67·8; H, 8·1; N, 9·9%.) Acidification of the alkaline extract and subsequent working up in the usual way gave 5:6-seco*cholestan*-3-*ol*-5:7-*dion*-6-*oic acid* (5·4 g.) as a colourless flaky glass, softening and melting indefinitely in the range 60–100°. (Found: C, 72·2; H, 10·1. $C_{27}H_{44}O_5$ requires C, 72·3; H, 9·8%.) Absorption at $\lambda > 220$ mμ. was very small. Reaction with boiling ethanolic 2:4-dinitrophenylhydrazine in the presence of HCl led to the formation of 5:6-seco-B-*norcholestan*-6-*al*-3-*ol*-5-*one* 2:4-*dinitrophenylhydrazone*, which crystallized from ethyl acetate-methanol in orange prisms, m.p. 164–166° decomp. (Found: C, 65·9; H, 8·5. $C_{32}H_{48}O_6N_4$ requires C, 65·8; H, 8·2%.) 5:6-seco-B-*Norcholestan*-6-*al*-3-*ol*-5-*one* 3:5-*dinitrobenzoate* was obtained from the keto acid and 3:5-dinitrobenzoyl chloride in dry pyridine-benzene. It formed an amorphous white powder, m.p. 128–129°, when precipitated from ethyl acetate by ethanol at −20°. (Found: C, 66·1; H, 7·7; N, 4·8. $C_{33}H_{46}O_8N_2$ requires C, 66·2; H, 7·7; N, 4·7%.)

The diketo acid (XII; R=OH) (3·0 g.) was allowed to stand in 10% (v/v) ethereal H_2SO_4 (50 ml.) for 20 hr. at room temperature. After washing the H_2SO_4 out with water, evaporation to dryness *in vacuo* gave 5:6-seco-*cholest*-3-*ene*-5:7-*dion*-6-*oic acid* (2·25 g.), yellow crystalline plates, m.p. 72°. (Found: C, 74·9; H, 9·7. $C_{27}H_{42}O_4$ requires C, 75·3; H, 9·8%.) $\lambda_{max.} = 220$ mμ.; $\epsilon = 11500$ in ethanol. Hydrogenation at atmospheric pressure was carried out in ethyl acetate in the presence of 5% $PdCl_2$ on charcoal, when the desired two atoms of hydrogen were taken up in 3 hr. From the alkaline extract of the product, a 30% yield of 5:6-seco*cholestan*-5:7-*dion*-6-*oic acid*, m.p. 57°, was obtained. It formed colourless plates when reprecipitated from dilute NaOH solution with dilute HCl. (Found: C, 74·8; H, 10·0. $C_{27}H_{44}O_4$ requires C, 75·0; H, 10·2%.)

Degradation of cholest-5-ene to 2-methylcyclohexanone

Finely powdered cholesterol (50·0 g.) was placed in a 250 ml. two-necked round-bottomed flask fitted with a calcium chloride guard tube and a small separating funnel. Purified $SOCl_2$ (40 ml.) was added rapidly with shaking, and the dark-green solution allowed to stand at room temperature for 18 hr. Excess $SOCl_2$ was removed at 45°/25 mm. Two crystallizations of the residue from acetone now gave cholesteryl chloride (43·6 g., 84% yield), m.p. 95°, of a high degree of purity. Cholesteryl chloride (40·0 g.) in boiling *n*-amyl alcohol (800 ml., freshly distilled) was treated with Na (approx. 40 g.) added in small lumps over 2 hr. The solution was heated under reflux for a further 1·5 hr. and then allowed to cool to room temperature overnight. After thorough washing with water (formation of an emulsion may be prevented by warming) to remove NaOH, the product was isolated by distillation of the *n*-amyl alcohol *in vacuo*. The white crystalline residue was recrystallized from ethanol, yielding pure cholest-5-ene (33·9 g., 93% yield), m.p. 92°.

Cholest-5-ene (10·0 g.) in *n*-hexane (100 ml.) was treated with a steady current of ozone until a dilute solution of Br_2 in acetic acid was no longer rapidly decolorized on mixing with a few drops of the *n*-hexane sclution. Evaporation to a small volume and fission with H_2O_2 as described for

cholesterol ozonide led to the isolation of 5:6-seco*cholestan*-5-*on*-6-*oic acid* (4·9 g.) as colourless plates of the *monohydrate*, m.p. 61°. (Found: C, 73·8; H, 10·7. $C_{27}H_{48}O_3.H_2O$ requires C, 74·3; H, 11·0%.) In a further experiment on the same scale, the *n*-hexane solution of the ozonide was evaporated *in vacuo* to small bulk, and reduction effected by shaking with Zn (10 g.) and acetic acid (70 ml.) for 12 hr. Normal isolation procedures gave 5:6-seco*cholestan*-6-*al*-5-*one* (10·1 g.) as a thick viscous oil. (Found: C, 76·8; H, 11·5. $C_{27}H_{46}O_2.H_2O$ requires C, 77·0; H, 11·4%.) Treatment with ethanolic 2:4-dinitrophenylhydrazine as before in the presence of HCl gave a 70% yield of the 2:4-*dinitrophenylhydrazone*, which crystallized from ethanol-(N-HCl) in clusters of bright-yellow prisms, m.p. 123°. (Found: C, 68·3; H, 8·8; N, 9·3. $C_{33}H_{50}O_5N_4$ requires C, 68·0; H, 8·6; N, 9·6%.) The same *seco*aldehyde (8·8 g.) in ethanol (90 ml.) was allowed to stand for 24 hr. with 7% ethanolic sodium ethoxide (10 ml.). The product was isolated as described for the analogous compound from cholesterol ozonide: 6-*formyl*-B-*norcholest*-5(6)-*ene* (8·0 g.) formed a lemon-yellow oil. $\lambda_{max.} = 253$ mμ.; $\epsilon = 6230$ in ethanol. The 2:4-*dinitrophenylhydrazone* crystallized from ethyl acetate in spherical clusters of fine orange hair-like needles, m.p. 235°. (Found: C, 70·2; H, 8·9; N, 10·0. $C_{33}H_{48}O_4N_4$ requires C, 70·2; H, 8·9; N, 9·9%.)

Ozonization of the noraldehyde (13·5 g.) was carried out as before, disappearance of the yellow colour being the criterion of a completed reaction. The subsequent working up this time revealed the presence of a large neutral fraction (5·65 g.) in addition to the 5:6-*seco*cholestan-5:7-dion-6-oic acid (6·6 g.), m.p. 57°, identical with the material previously obtained by reduction of the corresponding unsaturated diketo acid. The neutral fraction (6·1 g.) was purified by chromatography on alumina (194 g.) washed with methyl formate. Elution with benzene gave 5:6-seco*cholestan*-6-*al*-5:7-*dione* (3·5 g.) which crystallized from ethanol-water in colourless plates, m.p. 142–143°. (Found: C, 77·9; H, 10·8. $C_{27}H_{44}O_3$ requires C, 77·9; H, 10·6%.) The above diketo acid (5·0 g.) was heated under N_2 at 250° for 1·5 hr. The product was separated into neutral (2·3 g.) and acidic (mainly unchanged starting material) fractions. The neutral material was dissolved in light petroleum (b.p. 80–100°) and chromatographed on alumina (69 g.) washed with methyl formate. Elution with light petroleum (b.p. 80–100°) gave crystalline 5:6-seco-B-*norcholestan*-6-*al*-5-*one* (1·24 g.) which recrystallized from ethanol as colourless needles, m.p. 114°. (Found: C, 80·8; H, 11·0. $C_{26}H_{44}O_2$ requires C, 80·4; H, 11·3%.)

The crude neutral product (0·4 g.) from the ozonolysis of 6-formyl-*B*-norcholest-5-ene was heated at 450–500° with K_2CO_3 (1·5 g.) for 10 min. Neutralization and steam distillation of an aqueous suspension of the combined residue and distillate gave 2-methyl*cyclo*hexanone, isolated as the 2:4-dinitrophenylhydrazone. This derivative, recrystallized from ethanol, had m.p. 127–132°; an authentic specimen had a m.p. of 134°; the mixed m.p. was 131–133°. The yield was 0·028 g. (10%). A similar yield of 2-methyl*cyclo*hexanone was obtained by pyrolysing the diketo acid (X, R=H, 0·5 g.) at 250° for 1·5 hr., and then heating the crude residual product for 10 min. with K_2CO_3 (1·5 g.) at 450–500°. When degradations were being carried out on material labelled with ^{14}C, inactive 2-methyl*cyclo*hexanone was added to the steam distillate (after assay of a small portion by conversion to the 2:4-dinitrophenylhydrazone), and the 'diluted' material extracted into ether.

Degradation of 2-methylcyclohexanone

2-Methyl*cyclo*hexanone (10·15 g.) in conc. HCl (50 ml.) was cooled to 0°. NaN_3 (9·0 g.) was added in small portions with stirring during 5–10 min. Stirring was continued for 30 min., the solution was allowed to come slowly to room temperature, and then evaporated to dryness *in vacuo*. The residue was taken up in the minimum amount of water and made strongly alkaline with 50% KOH. Extraction into $CHCl_3$ (3 × 2 vol.) now removed all the product from the aqueous phase. The $CHCl_3$ solution was washed with a little water, dried over Na_2SO_4 and evaporated to dryness *in vacuo*, leaving a mixture of lactams (10·6 g., 92% yield; m.p. 70–75°). Three crystallizations from light petroleum (b.p. 80–100°) gave pure ϵ-amino-*n*-heptanoic lactam, m.p. 91°. (Found: C, 66·2; H, 10·3; N, 11·0. Calc. for $C_7H_{13}ON$: C, 66·1; H, 10·2; N, 11·0%.) The over-all recovery was only about 30%. A better yield of pure material (60–70%) was obtained by chromatography on alumina washed with methyl formate. The first fraction, eluted with light petroleum (b.p. 80–100°), contained only pure ϵ-amino-*n*-heptanoic lactam, but later fractions all proved to be mixtures. A method was not found for the isolation in a pure state of the other isomer, ϵ-amino-α-methyl-*n*-hexanoic lactam, although it was considerably concentrated both in the mother liquors from the first crystallization and in the later chromatographic fractions.

The crude lactam (10·0 g.) was boiled with 2N-HCl (80 ml.) for 1·5 hr., and the clear solution evaporated to dryness *in vacuo*. The product (14·25 g., 99%) solidified on standing; m.p. 112–120°. It crystallized from ethanol-ether in beautiful clusters of colourless rods; the pure ϵ-amino-*n*-heptanoic acid hydrochloride had m.p. 130–132°. (Müller & Krauss, 1932, give m.p. 131°. Found: C, 46·4; H, 8·9; N, 7·8. Calc. for $C_7H_{16}O_2NCl$: C, 46·3; H, 8·8; N, 7·7%.)

The crude amino-acid hydrochloride (7·5 g.) from the hydrolysis was dissolved in methanol (100 ml.) and heated under reflux for 12 hr. with methyl iodide (28·0 g.) and freshly prepared Ag_2O (from 45·5 g. $AgNO_3$). Filtration and evaporation to dryness *in vacuo* yielded a crude betaine (about 7 g., very deliquescent). However, after re-evaporation of a portion with dilute HCl, ϵ-*amino*-n-*heptanoic betaine hydrochloride* was obtained, and crystallized from ethanol-ether in clusters of colourless spears, m.p. 146–149°. (Found: C, 53·4; H, 10·1; Cl, 16·2. $C_{10}H_{22}O_2NCl$ requires C, 53·7; H, 9·9; Cl, 15·9%.) The betaine also formed a *picrate* which crystallized from water in magnificent yellow needles, m.p. 273–274° decomp. It was highly explosive, and attempts to obtain analytical figures were abandoned after the destruction of several pieces of micro-analytical apparatus.

One-fifth of the crude betaine obtained above was fused at 350° for 10 min. with solid KOH (10 g.). The residue was taken up in the minimum amount of water and acidified with H_2SO_4, when extraction with ether (3 × 2 vol.) removed all the fatty acids present except negligible amounts of acetic and propionic. The ethereal solution was dried and filtered. Neutralization with ethanolic NaOH, extraction of the salts into water, and evaporation to a small bulk left a concentrated solution of the sodium salts of the fatty acids which were separated by chromatography (see following paper). There were isolated: *n*-valeric (370 mg., 44%) *n*-butyric (47 mg., 6%), propionic (38 mg., 6%) and acetic acids (220 mg., 43%). (The yields are based on the crude amino-acid.)

The same sequence of reactions as that described in the previous paragraph was carried out on pure recrystallized ϵ-amino-*n*-heptanoic lactam, m.p. 91°. Liquid-vapour partition chromatography (James & Martin, 1952) of the final mixture of fatty acids showed them to be present in the following molar proportions: *n*-valeric and acetic 99% each, *n*-butyric and propionic 1% each. In a similar series, the mother liquor from the first light petroleum crystallization of the Schmidt reaction mixture was taken, and the crude product (m.p. 58–65°), obtained by evaporating to dryness *in vacuo*, was used as starting material. The following molar proportions of fatty acids were finally detected: *n*-valeric and acetic 67% each, *n*-butyric and propionic 33% each.

SUMMARY

1. A new method for the degradation of the ring structure of cholesterol is described. This involves the opening of ring *B* by oxidation and the splitting off of ring *A* as 2-methyl*cyclo*hexanone by pyrolysis.

2. Carbon atom 6 is obtained as carbon dioxide from the decarboxylation of 5:6-*seco*cholestan-5:7-dion-6-oic acid. From the further degradation of 2-methyl*cyclo*hexanone, carbon atoms 4 and 5 of cholesterol are obtained as acetic acid and carbon atoms 19, 10, 1, 2 and 3 as valeric acid; by a side reaction carbons 19, 10 and 5 were isolated as propionic acid accompanied by carbons 4, 3, 2 and 1 as butyric acid.

3. Two different ways are described for carrying out the early stages of the degradation: the first method starts from cholesterol ozonide, the second and more efficient method from cholest-5-ene.

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Studies of Cholesterol Biosynthesis

2. DISTRIBUTION OF ACETATE CARBON IN THE RING STRUCTURE

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It is now firmly established, largely on the basis of the work of Bloch and his collaborators (see Bloch, 1951) that acetic acid forms the primary building unit from which animal tissues synthesize cholesterol. Little & Bloch (1950) showed that both the side chain and the *cyclo*pentenophenanthrene nucleus are formed from acetate, and that of the twenty-seven carbon atoms of cholesterol fifteen originate from the methyl and twelve from the carboxyl carbon of acetate. A knowledge of the pattern according to which these acetate carbons are distributed in the sterol molecule might give valuable information about the intermediary stages of biosynthesis. Wüersch, Huang & Bloch (1952)

have already identified the distribution of acetate carbon in the side chain of cholesterol; their results are discussed later.

In a previous paper (Cornforth, Hunter & Popják, 1953*a*) we have described a new method for the partial degradation of the ring structure of cholesterol whereby carbon atoms 1, 2, 3, 4, 5, 6, 10 and 19 can ultimately be obtained each as carbon dioxide. This method has been applied to [^{14}C]-cholesterol synthesized by liver slices *in vitro* from [*carboxy*-^{14}C]acetate and [α-^{14}C]acetate respectively; the results, reported already in a preliminary form (Cornforth, Hunter & Popják, 1953*b*; Cornforth, 1953) are set out here in detail.

MATERIAL AND METHODS

Biosynthesis of [^{14}C]*cholesterol*

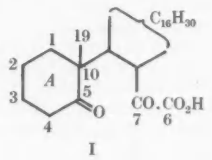
Slices (10 g.) made from the livers of young rats (50–60 g. body weight) were incubated at 37° for 6 hr. in 90 ml. of Krebs-Henseleit solution (pH 7·4) to which 0·3% glucose and either [*carboxy*-^{14}C]acetate (100 μc.) or [α-^{14}C]acetate (300 μc.) in a concentration of 0·01 M were added (Bloch, Borek & Rittenberg, 1946). At the end of incubation the contents of the flasks were made acid and the $^{14}CO_2$ was swept out with N_2 and trapped in NaOH. Then the contents of the flask were hydrolysed with 10 ml. of a saturated solution of KOH overnight on a hot-water bath. The unsaponifiable matter was extracted with light petroleum (b.p. 40–60°) and the cholesterol precipitated as the digitonide, which, after washing and drying, was decomposed by heating under reflux for 10 min. in 50 ml. of acetic acid to which 2 g. of inactive cholesterol were added. The hot solution was poured into a large volume of cold water and the precipitated cholesterol filtered off and washed free of acid on a Büchner funnel. The cholesterol was then crystallized from ethanol. The yield in terms of radioactivity was 2·5–3·5% from the labelled acetate; 7 μc. of [^{14}C] cholesterol derived from [*carboxy*-^{14}C]acetate and 13 μc. derived from [α-^{14}C]acetate were available for degradation.

Degradation of cholesterol

The detailed chemistry of the procedure has been described by us previously (Cornforth *et al.* 1953*a*); here only a few further pertinent details will be given. For the first stages of the degradation the [^{14}C]cholesterol samples were diluted with inactive cholesterol to 20 g. and converted to cholest-5-ene which served as the starting material for the further chemical manipulations.

Carbon atom 6 was obtained as barium carbonate from the decarboxylation of a sample of 5:6-*seco*-cholestan-5:7-dion-6-oic acid (I) by boiling with aniline.

Ring *A*, together with carbon atom 19, was isolated as 2-methyl*cyclo*hexanone and was diluted with unlabelled 2-methyl*cyclo*hexanone for further degradation, which gave a mixture of mainly acetic and *n*-valeric acid, and also from a side reaction a small amount of propionic and *n*-butyric acids. From the chemistry of the degradation of 2-methyl-*cyclo*hexanone given in our previous paper it is evident that the acetic acid contained carbon atoms 4 and 5 of cholesterol, the valeric acid carbon atoms



I

19, 10, 1, 2 and 3, the propionic acid carbon atoms 19, 10 and 5, and the butyric acid carbon atoms 4, 3, 2 and 1; in each acid the first-named carbon was the ω- and the last-named carbon the carboxyl-carbon (Fig. 1). The carbon-by-carbon degradation

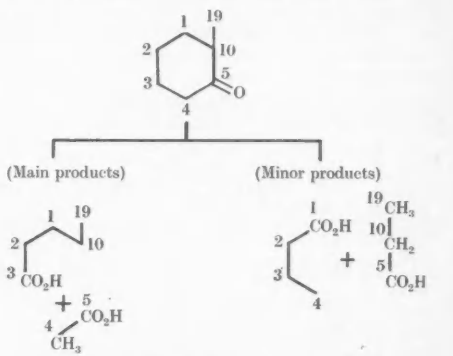


Fig. 1. Scheme of degradation of 2-methyl*cyclo*hexanone derived from ring *A* of cholesterol.

of either pair of acids, acetic and valeric, or propionic and butyric, should furnish, therefore, all the carbon atoms of ring *A* and the carbon (19) of the first angular methyl group of cholesterol. Since 80–90% of the fatty acids in the reaction mixture from the degradation was accounted for by acetic and valeric acid, these two were subjected to further degradation. The propionic acid was also degraded to obtain a check on carbons 19, 10 and 5; the agreement was very good.

*Separation of the fatty acids from degradation of 2-methyl*cyclo*hexanone.* The acetic and propionic acids were separated on a 'sulphuric acid' column (Martin & Webb, unpublished); the *n*-butyric and *n*-valeric acids, which were eluted together as the first band from this column, were separated on a modification of a buffered column described by Moyle, Baldwin & Scarisbrick (1948). Fig. 2 illustrates the complete separation of the four acids on the two columns; each measured 2·7 × 60 cm.,

equivalent to about 350 ml. bed, and could take a maximum load of about 500 mg. of the acids in one run. Both columns could be used repeatedly without deterioration.

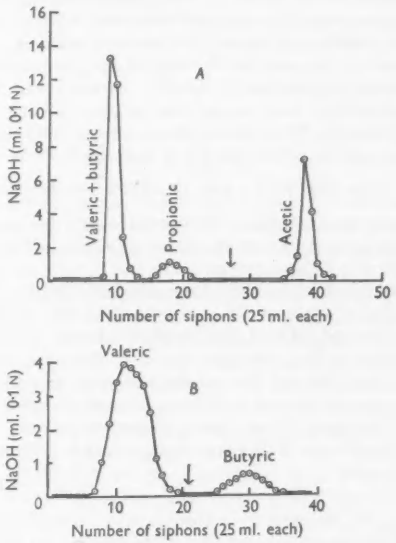


Fig. 2. Separation by chromatography of acetic, propionic, *n*-butyric and *n*-valeric acids obtained from the degradation of 2-methyl*cyclo*hexanone. The mixture of the acids was run first on column *A*, the packing of which consists of Hyflo Super-Cel (100 g.) with 0·5 N-H_2SO_4 (80 ml.) as the stationary phase. Moving phase: $CHCl_3$ changed to 10% butanol in $CHCl_3$ at arrow (Martin & Webb, unpublished). The first peak from column *A*, containing valeric + butyric acid, was resolved on column *B*. Packing: Hyflo Super-Cel (100 g.) with 2M-phosphate buffer (80 ml.) (2M-KH_2PO_4:2M-K_2HPO_4, 1:1, v/v), pH 6·8, as stationary phase. Solvents used were the same as for column *A* (modified from Moyle, Baldwin & Scarisbrick, 1948).

*Degradation of the acids derived from 2-methyl*cyclo*hexanone.* The *n*-valeric acid, diluted two- to four-fold, was split further into propionic acid (carbons, 19, 10 and 1 of cholesterol) and acetic acid (carbons 2 and 3 of cholesterol) by a general method previously described (Hunter & Popják, 1951) and the acids separated by chromatography on the 'sulphuric acid' column. It should be mentioned that approximately 25% of the valeric acid was recovered unchanged from the reaction.

The two propionic acids, the one obtained from the valeric acid and the other derived more directly from 2-methyl*cyclo*hexanone (carbons 19, 10 and 5) were oxidized with potassium dichromate in 15N-H_2SO_4 to carbon dioxide (=carboxyl carbon of propionic acid) and acetic acid. Forty ml. of 15N-sulphuric acid and 0·5 g. of potassium dichromate were used per millimole of propionic acid (Nahinsky & Ruben, 1941).

The acetic acid obtained from the various reactions was degraded by pyrolysis of the lithium salt at 380° *in vacuo* to give lithium carbonate and acetone as described by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949) for barium acetate. The carbon dioxide from the lithium carbonate (=carboxyl carbon of acetate) was liberated *in vacuo* with lactic acid and trapped in barium hydroxide. The iodoform (=methyl carbon of acetate) resulting from the treatment of acetone with sodium hypoiodite was oxidized to carbon dioxide by the method of Van Slyke, Plazin & Weisiger (1951). Corrections have been made for the small errors involved in the degradation of isotopic lithium acetate by pyrolysis (Popják, Hunter & French, 1953). The steps of the degradation described above and which gave each carbon atom individually, are summarized in Fig. 3.

Assay of ^{14}C. All samples of CO_2 were collected as $BaCO_3$ and their radioactivities measured, in 'infinite thickness' samples of 2 cm.² area, with an end-window Geiger-Müller counter. Samples of the organic substances (cholest-5-ene, 2-methyl*cyclo*hexanone 2:4-dinitrophenylhydrazone and the sodium and silver salts of fatty acids) were also assayed for radioactivity and served as standards to determine dilution factors correctly. All counts (after making the usual corrections) were calculated to counts per carbon atom

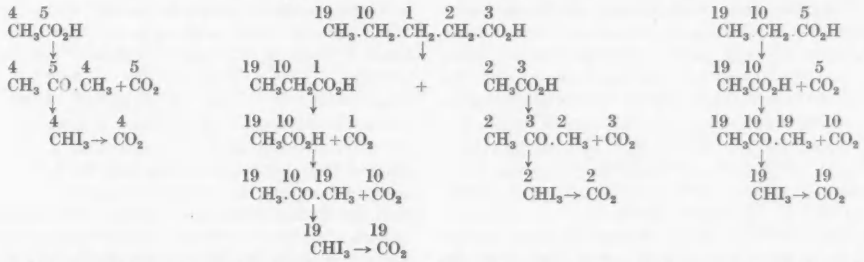


Fig. 3. Flowsheet showing steps of carbon-by-carbon degradation of acetic, *n*-valeric and propionic acids derived from 2-methyl*cyclo*hexanone. The figures above the carbon atoms indicate the original position of these atoms in the cholesterol molecule.

in infinite thickness of 2 cm.² area by multiplying the sample counts by the factor (mol.wt./12n), n being the number of carbon atoms in the substance. Dilution factors were also taken into account and all radioactive counts are referred to the activity of the cholest-5-ene as the starting material. The standard error of the counts was, of course, also multiplied by the same factors. The standard error of counts given is the combined error of sample and background counts. Thus if sample plus background counting rate is $(A \pm \sigma_1)$/min. and background count $(B \pm \sigma_2)$/min., then the sample count, $S = (A - B) \pm \sqrt{(\sigma_1^2 + \sigma_2^2)}$.

RESULTS AND DISCUSSION

The results are shown in Table 1. When [*carboxy*-^{14}C]acetate was the precursor of cholesterol, only carbon atoms 2, 4, 6 and 10 contained ^{14}C. We think that the relatively small differences found in the radioactivities of these carbons may be accounted for by experimental error. In cholesterol derived from [α-^{14}C]acetate carbon atoms 1, 3, 5 and 19 were highly labelled, but carbon atoms 2, 4, 6 and 10 contained also a significant amount of radioactivity; the specific activity of the latter group of carbons being about 20% of that of the former group. This latter result can probably be explained by the formation of [*carboxy*-^{14}C]acetate from [α-^{14}C]acetate via the tricarboxylic acid cycle before incorporation into cholesterol. Although some degree of redistribution of the isotopic carbon from the methyl-labelled acetate was expected, nevertheless the values observed were surprisingly high, for *in vivo* in the milk fatty acids of rabbits (Popják *et al.* 1953) a much smaller redistribution was found. The results indicate that a very high percentage (about 40%) of the acetate in the liver slices must have passed through the tricarboxylic acid cycle before being used for cholesterol synthesis.

Table 1. *Radioactivity of carbon atoms of cholesterol synthesized by rat-liver slices from* $CH_3.{}^{14}COOH$ *and* $^{14}CH_3.COOH$

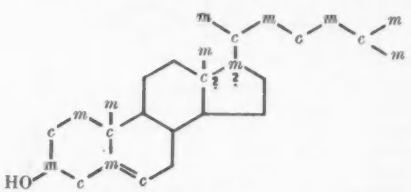
Cholesterol precursor ...	Counts/min./carbon atom in infinite thickness	
	$CH_3.{}^{14}COOH$	$^{14}CH_3.COOH$
Cholest-5-ene	756±6	1560±19
2-Methyl*cyclo*hexanone (C-19, -10, -1, -2, -3, -4, -5)	723±6	1505±15
C-1	0	2020±260
C-2	1643±147	516±50
C-3	0	2430±110
C-4	1750±125	314±54
C-5	0	2460±109
C-6	1580±25	454±13
C-10	1710±130	550±30
C-19	0	2500±120

Little & Bloch (1950) concluded that twelve carbon atoms of cholesterol are derived from the carboxyl and fifteen from the methyl carbon of cholesterol. Our results are in agreement with this conclusion. In our cholesterol sample derived from [*carboxy*-^{14}C]acetate the average specific activity of the radioactive carbon was 1671 counts/min. Assuming twelve carbon atoms derived from carboxyl in the molecule, the specific activity of the total carbon of the cholesterol should be 742 counts/min. Similarly, when [α-^{14}C]acetate was the precursor of the labelled cholesterol the mean specific activity of the highly labelled carbons was 2345 counts/min. and of the less active carbons 450 counts/min. The calculated specific activity of the total carbon of cholesterol in this case is

$$(15 \times 2345 + 12 \times 450)/27 = 1503 \text{ counts/min.}$$

Both values, 742 and 1503 counts/min., are in very close agreement with the observed figures of 756 and 1560 counts/min. respectively.

Wüersch *et al.* (1952) have recently identified the origin of the carbons in the *iso*octyl side chain of cholesterol. Also Little & Bloch (1950) have previously concluded that the two angular methyl carbons, 18 and 19, are derived from the methyl carbon of acetate and have tentatively identified carbon atom 13 as coming from the carboxyl and carbon atom 17 from the methyl carbon of acetate.



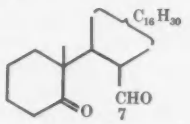
II

The results of Bloch and his collaborators combined with ours give the distribution pattern of acetate carbon in cholesterol as shown in the formula (II), in which *c* denotes a carbon derived from the carboxyl carbon and *m* a carbon derived from the methyl carbon of acetate. Wüersch *et al.* (1952) drew attention to the 'similarities in some structural features between the steroids and various natural products composed of isoprene units' and suggested the possibility that acetate is converted to cholesterol through C_5 units 'formally related to isoprene'. They cite in support of this thesis the observation of Bonner & Arreguin (1949) that acetate is utilized in the synthesis of natural rubber by isolated guayule leaves. Bonner & Arreguin suggest that an isoprene unit may be formed from acetate through a series of reactions involving first the formation of acetoacetic acid followed by decarboxylation to acetone and the condensation of the latter with acetate. According to this scheme the carbons of acetic acid should be distributed in the isoprenoid unit as shown in formula (III),

c indicating the carboxyl and *m* the methyl carbon of acetate. There is sufficient evidence to show that acetoacetic acid is indeed an intermediate in the biosynthesis of cholesterol (Brady & Gurin, 1951; Zabin & Bloch, 1951*a*, *b*). Bloch (1951) has recently revived the old hypothesis that squalene is an intermediate in the biosynthesis of cholesterol

III

(Channon, 1926; Robinson, 1934). Furthermore, Langdon & Bloch (1952) gave experimental support to this hypothesis; they fed to rats 0·5 g. of squalene together with 0·5 mc. of [^{14}C]acetate per 100 g. body weight for 2 days and isolated from the liver of the animals 35 mg. of squalene which was radioactive. After this labelled squalene had been fed to rats, in spite of the poor absorption of squalene, about 8% of the total dose was recovered in body cholesterol. This is the highest degree of utilization of any precursor for cholesterol synthesis as yet recorded. Bloch (1951) suggested that squalene, synthesized from the condensation of isoprenoid units in which acetate carbons are distributed according to the pattern shown in formula (III), may be folded to provide the entire carbon skeleton of cholesterol after the formation of carbon-to-carbon cross-linkages and the elimination of three methyl carbons. He predicted, on the basis of this hypothesis, a certain distribution pattern of acetate carbons in cholesterol. The results obtained by Wüersch *et al.* (1952) and by us are so far in complete harmony with this hypothesis (cf. formula I). It should be pointed out that the ultimate proof for this hypothesis will in all probability be provided when the origin of carbon atom 7 of cholesterol is established. The manner in which, according to Bloch (1951), the squalene molecule must be folded in the formation of cholesterol requires that the point of symmetry of the squalene molecule lie between carbons 6 and 7 of cholesterol and that both these carbons originate from the carboxyl carbon of



IV

acetate. We have shown this to be true of carbon atom 6. Unfortunately, all our attempts to isolate carbon atom 7 by the decarbonylation of the keto-aldehyde (IV) have so far failed. Further work is being planned to isolate this and other carbon atoms of the ring structure of cholesterol.

SUMMARY

1. [^{14}C]Cholesterol synthesized by rat-liver slices from [*carboxy*-^{14}C]acetate and [α-^{14}C]acetate has been degraded partially.

2. It is concluded that carbon atoms 2, 4, 6 and 10 are derived from the carboxyl carbon and carbon atoms 1, 3, 5 and 19 from the methyl carbon of acetate.

3. The bearing of these results on hypotheses concerning the formation of cholesterol from isoprenoid units via squalene is discussed.

The technical assistance of Miss Christine Connolly is gratefully acknowledged.

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Analysis of Lipids by Spot Tests on Filter-paper Disk Chromatograms

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There is a need in lipid research for a rapid and sensitive method for separating and identifying the lipid components of the mixture obtained by extraction of tissues. For this purpose column chromatography has not been successful (Zechmeister, 1950; Rathmann, 1944; Trappe, 1940*a*, *b*, 1941), although this technique has been useful in the purification of certain lipids (Thannhauser & Setz, 1936; Klenk, 1942; Hanahan, Turner & Jayko, 1951). Some of the water-soluble hydrolysis products of lipids were recently investigated by means of paper chromatography (Brante, 1949; Levine & Chargaff, 1951*a*, *b*; Munier, 1951).

We present here a rapid chromatographic technique for the analysis of lipids in quantities of 10^{-2} μmoles or less, using single filter-paper disks. The disk technique has independently been applied by others to the analysis of dyes (cf. Williams, 1947); to cations (Pollard & McOmie, 1951); and to sugars (Bersin & Muller, 1952).

Bevan, Gregory, Malkin & Poole (1951) and Hecht & Mink (1952) have recently reported paper-chromatographic techniques for the analysis of phosphatides.

METHODS

The tissue extract

Extraction of lipids from freeze-dried tissue can be effected as described previously for blood and plasma (Hack, 1947). Because kephalins, present in these extracts, are firmly adsorbed on filter paper it is necessary to use alundum or sintered-glass thimbles in order to avoid selective loss of these phosphatides. For this reason, extracts prepared by other procedures should not be filtered through filter paper but should be clarified by centrifugation or by filtration through sintered glass.

A $CHCl_3$: methanol (4 : 1, v/v) extract of freeze-dried tissue may in general be presumed to contain the total lipid; a benzene extract may be presumed to contain all lipid not bound to protein. This notion is based on the observation that polar solvents are required to extract lipid from lipoprotein (cf. Chargaff, 1949; Hack, 1947). Acetone extracts neutral fat, cholesterol and certain other non-phosphatide lipids; the lipid remaining in the tissue can be removed by $CHCl_3$: methanol and thus represents the acetone-insoluble fraction. In this way a convenient preliminary fractionation can be effected to precede chromatographic analysis. Experience has shown wet tissues to be less distinctly fractionated according to this scheme, possibly because of the influence of tissue water and water-soluble compounds on lipid solubilities.

Preparation of the chromatogram

The chromatograms were made with Schleicher & Schuell no. 576 or with Whatman no. 1 filter-paper disks (5·5 cm.) previously extracted with $CHCl_3$: methanol (4 : 1, v/v) and dried. The centre of the filter paper was indicated by a dot made with a pencil. With the filter paper supported on the rim of a 50 mm. Petri dish, 25 μl. of a $CHCl_3$: methanol, benzene or acetone solution containing 10^{-2} μmoles of lipid were added slowly with a Carlsberg pipette to the centre of the paper, confining the diameter of the sample spot to about 8 mm.; the solvent was evaporated by a current of nitrogen. Portions of $CHCl_3$: methanol (25 μl.) were then added until the lipid was spread to a ring 10 mm. in diameter (indicated by a pencil mark); two such portions usually sufficed. Finally, 25 μl. portions of the desired eluant were added at such a rate that the diameter of the wet spot increased about 2 mm./sec.; three such portions were generally sufficient to effect as complete separation of the lipid components as could be accomplished by the eluant chosen. The total time required for these operations was of the order of 5 min. When the eluant had been evaporated by a current of N_2, appropriate tests (see below) showed sharply defined concentric rings of the separated lipids. Often more complete separation could be effected by using two or more eluants.

It was frequently found desirable to cut the filter-paper chromatogram into two or more sectors and to test each piece with a different reagent in order to characterize the sample fully (cf. Figs. 1 and 2).

We have found it unnecessary to control the atmospheric conditions of the laboratory beyond avoiding strong air currents which may evaporate the solvent too rapidly.

An example. A 0·2 ml. sample of blood serum from a fasting human subject was freeze-dried and extracted with 1 ml. of $CHCl_3$: methanol at room temperature during 1 hr. The extract was then collected by filtering through a small medium-porosity sintered-glass filter. Two 25 μl. portions of this clear, pale-yellow extract were applied to a filter paper as described above. 25 μl. of $CHCl_3$: methanol were then added, bringing the lipid to a ring 10 mm. in diameter. After removal of the $CHCl_3$: methanol by a current of N_2 three 25 μl. portions of methanol were applied, not allowing the diameter of the wet spot to exceed 30 mm. After drying with N_2 the chromatogram was cut in half; one half was tested for choline by the dichromate-haematein method and the other treated with Nile blue. The result is schematized in Fig. 1*A*. A better separation was effected by eluting first with four 25 μl. portions of acetone to diam. 40 mm., drying, and then eluting to diam. 30 mm. with methanol as shown in Fig. 1*B*.

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In a similar manner a mixture of purified lipids can be separated as indicated in Fig. 2.

Eluants. The following organic solvents, dried by distillation over anhydrous $CaSO_4$, were found to be most generally useful as eluants because of their differential solvent action: acetone, benzene, $CHCl_3$ and methanol. Others, which could probably profitably be employed are pyridine, ethyl acetate, carbon disulphide and dichloroethylene.

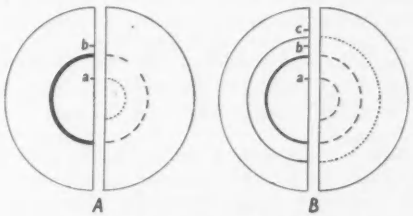

Fig. 1. Chloroform-methanol (4 : 1, v/v) extract of human blood serum eluted to *a* (*A* and *B*) with $CHCl_3$: methanol, to *b* (*A* and *B*) with methanol and to *c* (*B*) with acetone. The left half was treated for the detection of choline by the dichromate-haematein technique and the right half was treated with Nile blue. The black ring indicating choline is shown as a solid line in the figure; the red Nile-blue ring as a dotted line and the blue Nile-blue ring as a dashed line.

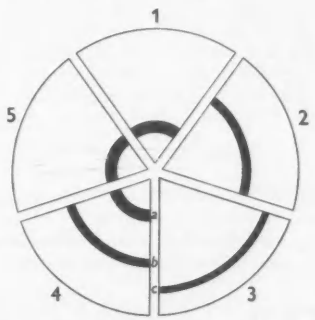

Fig. 2. Mixture of phosphatidyl serine (*a*), hydrolecithin (*b*), and glyceryl palmital (*c*) eluted by methanol to distance *c*. The five sectors were treated separately for the detection of: 1, amine; 2, choline; 3, acetal; 4, phosphate; 5, unsaturation.

Spot tests

Tests for choline lipids. (1) When the chromatogram is immersed, without previous hydrolysis, in aqueous 0·05 M-reinecke salt for 2 or more hours at room temperature, a pink ring appears at the site of the choline lipid (cf. Baer & Kates, 1950; Hack, 1946). Thorough rinsing in water removes the excess reagent and when dry the chromatogram may be retained as a permanent record. (2) Immersion for 1 min. in 0·0001 M-I_2 in aqueous KI at room temperature produces a transient yellow-brown colour (cf. Brante, 1949). (3) Reaction of chromatograms with aqueous 0·1 M $K_2Cr_2O_7$ at 60° for 1 hr. results in a faint brownish ring at the site of the choline lipid. This ring can be intensified by reacting with a freshly prepared solution of haematein (a mixture of 25 mg. haematoxylin, 50 ml. water and 0·2 ml. 30% (w/v) H_2O_2 heated to 60° for 10 min.) for 10 min. at 60°. The more prominent blue-grey chromium lake of haematein appears at the site of the brownish ring (cf. Baker, 1947). Excess reagent must be removed with water at each step. Similarly, aqueous 0·005 M-phosphomolybdic acid (cf. Levine & Chargaff, 1951 *a*, *b*) can be used; in this case a blue-lavender haematein lake is formed.

Test for amine lipids. Wetting the filter paper with aqueous 0·005 M-ninhydrin and drying for 15 min. at 105° results in a lavender ring at the site of lipids with a free NH_2 group.

Test for acetal lipids (plasmalogens). Hydrolysis of the acetal bond in the presence of aqueous 0·005 M-$HgCl_2$ at room temperature for 1 min. exposes the carbonyl group for reaction with fuchsin-sulphurous acid or with aqueous 2:4-dinitrophenylhydrazine. The excess fuchsin-sulphurous acid is removed with aqueous 0·05 M-SO_2 and the 2:4-dinitrophenylhydrazine with water (cf. Hack, 1952).

Test for phosphoric ester. Phosphate is detected as a blue ring when chromatograms are exposed to acid ammonium molybdate followed by H_2S according to the method of Hanes & Isherwood (1949). In this test sphingomyelin reacts more slowly than glycerophosphatides.

Test for cholesterol. Exposure of chromatograms to a 1 : 1 (v/v) mixture of acetic acid and H_2SO_4 (cooled to 20°) results in the appearance of a pink ring at the site of cholesterol.

Test for glycolipids. Glycolipids (cerebrosides, etc.) can be detected by oxidation with aqueous periodic acid and visualization of the resulting aldehyde by fuchsin-sulphurous acid as shown by Morrison & Hack (1949). The acetal lipids also give this test but can be differentiated from glycolipid by the plasmal reaction. However, some acetal lipids have elution characteristics similar to the glycolipids and have existed resolution by the eluants mentioned.

Test for unsaturation. Lipids containing unsaturated fatty acids and aldehydes appear as a brown to black ring after exposure for 5 min. to OsO_4 vapours.

Other tests. Although the mechanism of their reaction is not known, both aqueous 0·01 M-Biebrich scarlet and 0·01 M-Nile blue in 0·1 M-H_2SO_4 were also used. Biebrich scarlet was found to be a useful stain for choline lipid. In general, a blue colour with Nile blue seems to indicate a strong acid function as in cardiolipin, phosphatidyl serine, etc., and a pink colour, a strongly lipophilic compound as in free fatty acid, fatty aldehyde, neutral fat, etc.

RESULTS

Some of the results with a variety of purified lipids appear in Table 1. In addition to the observations given in this table, it was found that with iodine, a transient yellow-brown ring appeared at the site of phosphatides, which persisted longer when the compound contained choline. Both the phosphatidyl serine and the diphosphoinositide were shown to contain a material eluted by acetone and staining pink with Nile blue, presumably free fatty acid. In addition, the phosphatidyl serine contained a second ninhydrin-positive substance (not the intact phosphatide) elutable by acetone. We believe these

Table 1. *Spot-test reactions of various lipids*

(Compounds 1 and 12 were obtained through the generosity of E. Klenk; 3, 5 and 7 from S. J. Thannhauser; 4 and 8 from E. Baer; 6 and 11 from M. C. Pangborn and the Sylvana Chemical Co.; 9, 10 and 15 from J. Folch; 19 was a Hormel Foundation product; 20 and 21 from Armour and Co.; 22, an Eastman Kodak product; 2, 13, 14, 16, 17, 18 were prepared by the author. Preparations 2, 13, 14 were described earlier (Hack, 1946, 1947). The ganglioside (16) was isolated from human brain by the procedure of Klenk (1941) and the glycerylpalmital (18) was synthesized according to Bersin, Moldtman, Nafziger, Marchand & Leopold (1941). The cholesterol (17) was isolated from an acetone extract of human brain and was purified by repeated recrystallization from methanol. For experimental details see text. +, Positive; 0, negative.)

Compound	Reinecke salt	Potassium dichromate-haematein	OsO_4	Ninhydrin	Plasmal	Phosphate
1. Sphingomyelin (brain)	+	+	+	0	0	+
2. Sphingomyelin (brain)	+	+	+	0	0	+
3. Sphingomyelin (lung)	+	+	+ (slight)	0	0	+
4. Dipalmityllecithin (synthetic)	+	+	0	0	0	+
5. Dipalmityllecithin (lung)	+	+	0	0	0	+
6. 'Lecithin' (beef-heart)	+	+	+	0	+	+
7. Acetal phosphatide (brain)	0	Brown (slight)	+ (slight)	+	+	+
8. Phosphatidyl ethanolamine (synthetic)	0	Brown (slight)	+	+	0	+
9. Phosphatidyl serine (brain)	0	Brown (slight)	+	+	0	+
10. Potassium diphosphoinositide	0	0	.	.	0	+
11. Cardiolipin (beef-heart)	0	0	+	0	0	+
12. Phrenosin (brain)	0	0	0	0	0	0
13. Phrenosin (brain)	0	0	0	0	0	0
14. Kerasin (brain)	0	0	0	0	0	0
15. Sulphatide (brain)	0	Brown (slight)	.	0	0	.
16. Ganglioside (brain)	0	.	.	+	0	.
17. Cholesterol (brain)	.	0	0	.	.	0
18. Glycerylpalmital (synthetic)	.	0	0	.	+	0
19. Oleic acid	.	0	+	.	0	0
20. Palmitic acid	.	0	0	.	0	.
21. Stearic acid	.	0	0	.	0	.
22. Choline HCl	+	+	.	0	0	.

observations to indicate contamination by products of auto-oxidation.

The beef-heart 'lecithin' was separable from a ninhydrin-positive plasmalogen with methanol. By elution with chloroform, benzene, or acetone, this 'lecithin' yielded two choline components, both plasmalogens, hitherto undescribed. Preliminary experiments have shown glycerylphosphorylcholine and higher fatty aldehyde to be present in good yield after hydrolysis in 50 % (v/v) aqueous ethanol containing 0·001 M-$HgCl_2$ during 3 hr. at 20°. A detailed analysis of these choline plasmalogens is being undertaken. To avoid confusion the original name given to this substance (cf. Pangborn, 1941, 1945) is retained (in inverted commas), although our experiments indicate that the major component probably could be considered to be acetal-phosphatidyl choline. The multiplicity of plasmalogens has recently been demonstrated by Lovern (1952) who applied the technique of countercurrent distribution to the separation of phosphatides from ox brain.

The lecithins were separable from sphingomyelins by elution with chloroform and from kephalins by methanol. Sphingomyelins were eluted more rapidly by methanol than were cerebrosides. Plasmalogens were separable by acetone and by methanol.

Exposure of chromatograms to 0·1 M-KOH at room temperature for 20 hr. followed by thorough washing in water and extraction by acetone left only the sphingomyelins, which were demonstrable with dichromate-haematein.

In a preliminary study, several animal tissues (heart, spleen, anterior pituitary, lung, adrenal, kidney, mammary gland, spinal cord and blood serum) were examined by this technique; the extract from 1 mg. of freeze-dried tissue was more than sufficient for analysis. By the use of eluants, each of these tissues was shown to contain two or more plasmalogens, one or more of which was acetone-soluble and therefore probably not phosphatide. Also, by assay of benzene and chloroform-methanol extracts (cf. Hack, 1948), it was possible to estimate the proportion of protein-bound lipids in these tissues.

DISCUSSION

It should be emphasized here that the eluants used and the order of their use must be empirically determined for each tissue so as to obtain the best separation of the lipids in a given extract and that our procedure is to be regarded only as preliminary and to be modified as required.

R_F values (in terms of radial distance) were not determined for any of the compounds studied and are not requisite for its use as an empirical tool as described. This technique has served as a reliable test for the homogeneity of isolated lipids, and in some cases has permitted characterization of the contaminant.

The small quantities of tissue required made it practical to use the technique as an adjunct to histochemical studies of lipids, especially with plasmalogens (Hack, 1952), as it affords a means of verifying the presence of the lipid in question. Efforts are being made to apply some of the tests described to the *in situ* identification of lipids in cells and portions of cells. However, great caution must be exercised because some of the colour reactions described above are given by a variety of non-lipid nitrogenous substances (Guggenheim, 1920) occurring as constituents of proteins.

SUMMARY

1. A technique is presented for the examination of lipids in quantities of 10^{-2} μmoles by means of spot tests applied to filter-paper chromatograms. This technique permits the separation and characterization of lipid mixtures both in tissue extracts and in separated lipids.

2. It has become evident that several plasmalogens, detected by their elution characteristics, are present as constituents both of separated lipids and of tissues.

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Studies Using Radioactive Sulphur on Ergothioneine Formation in the Pig

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Until recently, blood erythrocytes were the only known source of ergothioneine in the animal body, and so far all studies on the formation of ergothioneine have been confined to blood. In experiments on the pig, rabbit and rat it has been found that certain diets, although themselves devoid of ergothioneine, can influence the level of ergothioneine in the blood (Eagles & Vars, 1928; Potter & Franke, 1935; Hunter, 1951; Spicer, Wooley & Kessler, 1951). Ergothioneine added to the diet was found to be incorporated into the blood of rats and rabbits (Spicer *et al.* 1951; Heath, Rimington, Searle & Lawson, 1952) and synthetic [^{35}S]ergothioneine fed to rats was shown by Heath (1953) to find its way into the blood and tissues.

Leone & Mann (1951) and Mann & Leone (1953) recently brought to light a second source of ergothioneine in the animal body, when they discovered that ergothioneine is a normal constituent of boar semen, and is secreted by the seminal vesicles. Unlike blood, the vesicular secretion was shown to contain ergothioneine as an extracellular constituent and in a remarkably high concentration.

The present paper reports the results of studies on the formation of both seminal and blood ergothioneine, obtained after feeding to boars ^{35}S-labelled inorganic sulphate, thiolhistidine, methionine and ergothioneine. It was found that neither [^{35}S]sulphate nor [^{35}S]thiolhistidine acts as a source of sulphur for ergothioneine. On the other hand, both [^{35}S]methionine and [^{35}S]ergothioneine were shown to be capable of labelling the body ergothioneine.

MATERIALS AND METHODS

Experimental animals. Two boars were used, *A* and *B*, both from an inbred strain of 'Large Whites' maintained at the Animal Research Station, Cambridge. At the start of the experiment, boar *A* was 3 years old, and weighed 220 kg. and boar *B* was 1 year old and weighed 150 kg. The boars were fed twice daily, the daily ration consisting of approximately 3 kg. of balanced meal mixture. Regular removal of faeces was carried out, and particular care taken at all times to avoid contamination of food with excreta.

Collection of semen, blood and urine. The semen was collected by means of an artificial vagina (Walton, 1945), usually at weekly intervals. As a rule, one complete ejaculate was collected on each occasion. The volume of the ejaculates varied from 200 to 600 ml., the density of semen from 50000 to 200000 sperm/μl. After removal of the gel, the semen was separated by centrifugation into sperm and seminal plasma, the sperm washed with Ringer solution, and the washing fluid added to the seminal plasma. Both the sperm and the seminal plasma were then treated with 3 vol. of ethanol, centrifuged after 1 hr., and thus separated into fractions, referred to hereafter as the ethanol-insoluble and ethanol-soluble portions; ergothioneine is confined entirely to the ethanol-soluble portion of the seminal plasma (Mann & Leone, 1953). The ethanolic extracts, both from the sperm and the seminal plasma, were concentrated *in vacuo*, at pH 6, to one-tenth of the original volume of semen. The ethanol-insoluble portions from sperm and plasma were washed with ethanol and dried.

Blood was collected from the auricular vein, in the presence of heparin. Urine was collected into a glass vessel placed outside the pen and connected by a rubber tube to a plastic funnel enclosing the prepuce and attached to the body by means of a plastic apron. In this manner, it was possible to obtain clean urine.

^{35}S-labelled compounds and their administration. $H_2{}^{35}SO_4$ and [^{35}S]methionine were obtained from the Atomic Energy Research Establishment, Harwell, and the Radiochemical Centre, Amersham. [^{35}S]Thiolhistidine and [^{35}S]ergothioneine were synthesized by the method of Heath, Lawson & Rimington (1951) using 25 g. $KCN^{35}S$ containing 10 mc. ^{35}S obtained from the Radiochemical Centre, Amersham. The radioactive compounds were administered to the boars in the form of dilute aqueous solutions added to their food. Care was taken to ensure that all the food was eaten by the animals.

Determination of total ^{35}S in tissues. The following quantities were used for wet oxidation: whole blood, 2 ml.; blood plasma, 5 ml.; blood cells, previously washed seven times with 0·9% (w/v) NaCl, 4 ml.; seminal plasma, ethanol-soluble portion, 3 ml., ethanol-insoluble portion, 0·5 g.; sperm, ethanol-soluble portion 5 ml., ethanol-insoluble portion

* Animal Health Trust Scholar.
† British Council Scholar.

0·5 g.; urine 6 ml. All samples were refluxed for 18 hr. with 15 ml. of a mixture of 1 vol. 60% (w/v) $HClO_3$ + 3 vol. 70% (w/v) HNO_3 in 50 ml. Kjeldahl flasks containing 0·1 g. Cu wire. The solutions were evaporated to dryness, the residue redissolved in 4 ml. 6 N-HCl and again evaporated to dryness, redissolved in 15 ml. boiling water with addition (if necessary to effect complete solution) of 0·1 ml. 6 N-HCl. Carrier H_2SO_4 (≡10 mg. $BaSO_4$) was added to the solutions obtained from ethanol-soluble portions of seminal plasma and semen. The total sulphate ions were then precipitated as $BaSO_4$, collected and washed in tared 10 ml. centrifuge tubes and, after drying at 105° and weighing, samples were transferred with 50% (v/v) ethanol to tared nickel planchets. The activity was determined with an end-window counter.

Fractionation of urinary sulphur

Boar urine is normally devoid of ergothioneine (Mann & Leone, 1953). However, as a result of feeding, particularly with [^{35}S]ergothioneine, it became necessary to include determinations of bromine-oxidizable S in the urine. The fractionation was carried out as follows:

Free sulphate. The free sulphate was precipitated as $BaSO_4$ from 10 ml. urine.

Ethereal sulphate. Urine (50 ml.) was treated with 20 ml. 3 N-HCl and 10 ml. 5% (w/v) $BaCl_2$ solution and filtered through a Whatman no. 42 filter paper. Carrier H_2SO_4 (≡25 mg. $BaSO_4$) was added to the filtrate, and the mixture boiled for 30 min. The $BaSO_4$ was collected as described above for total S.

Bromine-oxidizable S. To the supernatant liquid, filtered through a Whatman no. 42 filter paper, were added carrier H_2SO_4 (≡25 mg. $BaSO_4$) and 2 ml. Br_2; after 30 min., excess Br_2 was removed by boiling and the $BaSO_4$ collected as previously described.

All the $BaSO_4$ fractions obtained from urine were washed with 2 N-NaOH.

Fractionation of sulphur in seminal plasma

As shown by Mann & Leone (1953), the bulk of the non-protein sulphur present in the vesicular secretion of the boar can be accounted for as ergothioneine. In the whole semen, however, as ejaculated, the vesicular secretion constitutes no more than 25% of the total volume (McKenzie, Miller & Bauguess, 1938), the remaining part being made up by sperm and various secretory fluids including those of epididymis, prostate and Cowper's glands. It was therefore necessary to carry out a complete analysis of the various sulphur fractions.

Free and ethereal sulphate. To the ethanol-soluble portion of seminal plasma (15 ml.) were added 35 ml. carrier H_2SO_4 (≡25 mg. $BaSO_4$), 10 ml. 3 N-HCl and 5 ml. 5% (w/v) $BaCl_2$ and the mixture was boiled for 30 min. The $BaSO_4$ was collected as described above.

Bromine-oxidizable S. The supernatant liquid thus obtained was filtered through a Whatman no. 42 paper, and treated with Br_2 as described for the corresponding determination in urine. In this case the addition of carrier H_2SO_4 was unnecessary.

RESULTS

Experiments with $H_2{}^{35}SO_4$

Before carrying out the actual feeding experiment, proof had to be obtained that $H_2{}^{35}SO_4$ added to ejaculated semen *in vitro* does not confer any radioactive properties on seminal ergothioneine. A sample (1 ml.) of a solution containing 0·05 μc. $H_2{}^{35}SO_4$ was added to 90 ml. boar semen, the mixture was centrifuged 6 hr. later, and the sperm and seminal plasma fractionated into the ethanol-soluble and ethanol-insoluble portions. As can be seen from Table 1, some $^{35}SO_4$ was carried down with the ethanol-insoluble portions of both sperm and seminal plasma, but the bromine-oxidizable sulphur fraction was completely free from radioactivity. An additional control experiment was carried out in which a solution containing a mixture of pure ergothioneine and $H_2{}^{35}SO_4$ was separated into inorganic and bromine-oxidizable sulphur fractions. The latter was not radioactive (Table 1).

Table 1. *Radioactivity of* $BaSO_4$ *prepared by the fractionation of* (*a*) *a mixture of boar semen and* $H_2{}^{35}SO_4$ *and* (*b*) *a mixture of ergothioneine and* $H_2{}^{35}SO_4$

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Counts
(*a*) Seminal plasma	
Ethanol insoluble. Total S	31
Ethanol soluble. Total S	322
Ethanol soluble. Free + ethereal sulphate	1027
Ethanol soluble. Bromine-oxidizable S	0
(*b*) Sperm	
Ethanol insoluble. Total S	41
Free + etheral sulphate	457
Bromine-oxidizable S	0

Two experiments were carried out on boars fed with inorganic ^{35}S-labelled sulphate. In the first experiment, on boar *A*, 10 mc. carrier-free $H_2{}^{35}SO_4$ were diluted with water to 200 ml., and 50 ml. added to the daily ration on 4 successive days. Semen, blood and urine were then collected at weekly intervals. The results are recorded in Table 2.

It will be seen that the relatively high radioactivity of the free sulphate of urine and seminal plasma falls rapidly during the first 2 weeks. The slight radioactivity in the bromine-oxidizable fraction of the seminal plasma may be due not to ergothioneine but to possible traces of sulphide, thiosulphate or thiocyanate, although none of these was detectable by normal chemical means. The weight of barium sulphate obtained agreed with the calculated amount based on the colorimetric determination of ergothioneine; nevertheless, even a trace of the above-mentioned compounds with labelling comparable with that of the free sulphate fraction could account for the activity observed.

To decide whether or not the slight activity was due to ergothioneine, a second experiment was carried out on boar *B* in which 10 mc. carrier-free $H_2{}^{35}SO_4$ was diluted with water to 1 l. and 50 ml. of

Table 2. *Radioactivity of* $BaSO_4$ *prepared by the fractionation of the sulphur-containing constituents of boar urine, blood and semen obtained after feeding* 10 *mc.* $H_2{}^{35}SO_4$

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Days after last dose				
	3	10	17	24	31
	Counts				
Seminal plasma					
Ethanol insoluble. Total S	66	55	41	26	19
Ethanol soluble. Total S	400	211	77	74	55
Ethanol soluble. Free + ethereal sulphate	2350	1052	402	281	196
Ethanol soluble. Bromine-oxidizable S	105	69	60	53	53
Sperm					
Ethanol insoluble. Total S	—	45	51	37	32
Ethanol soluble. Total S	—	192	128	61	56
Blood					
Total S	330	83	47	25	18
Plasma. Total S	—	156	63	31	30
Cells. Total S	—	38	26	11	14
Urine					
Total S	5200	679	238	201	98
Free sulphate	5700	805	271	246	149
Ethereal sulphate	—	800	310	262	140
Bromine-oxidizable S	—	72	27	95	50

the solution administered daily on 20 successive days, so as to maintain a high level of $^{35}SO_4{}^{2-}$ activity in the animal for a longer period than in the first experiment. The results are set out in Table 3.

Table 3. *Radioactivity of* $BaSO_4$ *prepared by the fractionation of the sulphur containing constituents of boar semen obtained after the feeding of* 10 *mc.* $H_2{}^{35}SO_4$ *spread over a period of* 20 *days*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Seminal plasma (counts)	Spermatozoa (counts)
Ethanol insoluble. Total S	10	17
Ethanol soluble. Total S	31	83
Ethanol soluble. Free sulphate	148	6
Ethanol soluble. Ethereal sulphate	122	0
Ethanol soluble. Bromine-oxidizable S	12	0

Again a very slight activity was observed in the bromine-oxidizable fraction, actually lower than in the first experiment. It was proved that this activity was not due to ergothioneine which, after isolation as phosphotungstate, was entirely devoid of activity.

Experiments with [^{35}S]*thiolhistidine*

[^{35}S]Thiolhistidine (1 g.) giving 54×10^5 counts/min. (calculated from the counts at 'infinite thinness' using an end-window counter) was added to water (200 ml.) containing sufficient 2 N-hydrochloric acid to effect solution; 50 ml. portions were mixed with the food of boar *A* on 4 successive days. It should be borne in mind that the ame boar had received radioactive sulphate 5 weeks previously. The distribution of radioactivity can be seen from Table 4 which shows that orally administered thiolhistidine is rapidly excreted in an unchanged state in the urine; the bromine-oxidizable fraction of the first collection having high radioactivity. By the 15th day the radioactivity of this fraction was negligible. There was no incorporation of thiolhistidine into blood plasma or cells. The radioactivity of the seminal plasma on the 1st day after cessation of feeding could have been due to urinary contamination; less than 5 ml. of urine would have accounted for the observed activity of the semen (250 ml.). On the other hand, the possibility exists that as a result of feeding thiolhistidine, some of this compound may actually have passed through the male accessory glands and have been secreted into the seminal plasma. The prompt fall in activity of the bromine-oxidizable fraction of seminal plasma shows clearly that seminal ergothioneine has not been labelled and cannot therefore be derived from thiolhistidine.

Attention may be drawn to the rise in radioactivity of the ethanol-soluble portion of the sperm shown in the collections on the 8th and 15th day. This may be taken as indicating a slow but definite incorporation of ^{35}S into the spermatozoa; this radioactivity could have been derived from the $H_2{}^{35}SO_4$ administered to the same boar in the previously described experiment.

Table 4. *Radioactivity of* $BaSO_4$ *prepared by the fractionation of the sulphur-containing constituents of boar urine, blood and semen obtained after feeding* [^{35}S]*thiolhistidine*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Days after last dose				
	1	8	15	22	36
	Counts				
Seminal plasma					
Ethanol insoluble. Total S	19	10	17	10	5
Ethanol soluble. Total S	204	75	25	27	14
Ethanol soluble. Free + ethereal sulphate	189	115	45	36	20
Ethanol soluble. Bromine-oxidizable S	845	43	56	42	33
Sperm					
Ethanol insoluble. Total S	44	59	28	15	10
Ethanol soluble. Total S	88	504	490	23	1
Blood					
Total S	28	10	7	8	5
Plasma. Total S	36	14	4	7	3
Cells. Total S	12	7	5	6	8
Urine					
Total S	397	75	60	50	37
Free sulphate	153	92	67	62	40
Ethereal sulphate	198	102	66	56	42
Bromine-oxidizable S	8800	150	0	3	0

Table 5. *Radioactivity of* $BaSO_4$ *prepared by the fractionation of the sulphur-containing constituents of boar urine, blood and semen obtained after feeding* 1 *mc.* [^{35}S]*methionine*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

	Days after last dose						
	4	11	18	25	32	39	53
	Counts						
Seminal plasma							
Ethanol insoluble. Total S	223	277	215	194	198	153	126
Ethanol soluble. Total S	117	154	135	138	58	—	—
Ethanol soluble. Free + ethereal sulphate	136	160	67	56	55	—	—
Ethanol soluble. Bromine-oxidizable S	180	114	103	88	60	—	—
Sperm							
Ethanol insoluble. Total S	89	74	86	179	199	139	144
Ethanol soluble. Total S	17	12	12	111	80	62	52
Blood							
Total S	366	318	235	176	160	123	91
Plasma. Total S	561	387	282	175	162	111	88
Cells. Total S	153	174	152	174	160	157	103
Urine							
Total S	441	224	119	66	67	47	39
Free sulphate	531	231	116	72	64	57	41
Ethereal sulphate	219	162	119	59	53	41	20
Bromine-oxidizable S	21	24	20	0	0	0	0

Experiments with [^{35}S]*methionine*

[^{35}S]Methionine (13 mg.) containing 1 mc. ^{35}S was dissolved in 200 ml. water. Portions (50 ml.) of this solution were mixed with the food of boar *A* on 4 successive days starting 6 weeks after the termination of the thiolhistidine feeding. The results are set out in Table 5.

It can be seen that an appreciable radioactivity is present in the bromine-oxidizable fraction of seminal plasma as early as the 4th day after cessation of feeding even though the total amount of administered methionine was comparatively small, representing only 1 mc. radioactivity as against 10 mc. of the $^{35}SO_4$ used in the previously described experiment. Urinary contamination of the seminal plasma

can be excluded in view of the very low activity of the bromine-oxidizable fraction in the urine. Moreover, the level of labelling of the bromine-oxidizable fraction in seminal plasma was well maintained for several weeks. The fact that this activity is due to ergothioneine was confirmed by the actual isolation of ergothioneine from the semen.

The combined ethanol-soluble portions, containing 35 mg. ergothioneine from 380 ml. semen, collected on the 39th and 53rd day of the experiment recorded in Table 5, were treated with 4 vol. ethanol. The precipitate was discarded after centrifugation and the supernatant liquid was evaporated under reduced pressure nearly to dryness. The residue was

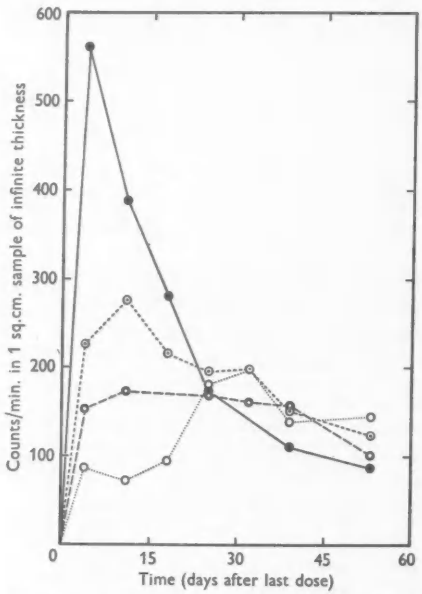

Fig. 1. Radioactivity of $BaSO_4$ prepared by fractionation of the sulphur-containing constituents of boar blood and semen obtained after feeding 1 mc. [^{35}S]methionine. ○···○, Sperm (ethanol-insol.); ⊙- - - -⊙, seminal plasma (ethanol-insol.); ○– – –○, blood cells; ●——●, blood plasma.

diluted to 50 ml. with water and made 0·2 N with respect to H_2SO_4. Phosphotungstic acid (10%, w/v) was added until precipitation was complete. The precipitated ergothioneine phosphotungstate was decomposed with $Ba(OH)_2$ solution; after concentration and crystallization from ethanol, 17·4 mg. ergothioneine were obtained. Although this ergothioneine preparation did not give a ninhydrin reaction, it was further purified by partition chromatography using Whatman no. 1 paper and butanol/acetic acid/water (50 : 10 : 40, v/v) mixture. The position of the ergothioneine was detected by spraying three thin strips cut from the edges and middle of each square, with a 0·4% (w/v) ethanolic solution of 2:6-dichloroquinone chloroimide. The ergothioneine-containing portions of the chromatograms were cut out and eluted with hot water.

The ergothioneine thus isolated gave a count of 37/min./sq.cm. at 'infinite thickness'. The absence of any ninhydrin-reacting material indicates freedom from amino-acids, and the method of isolation would have eliminated sulphates, sulphides, sulphites, thiocyanates and thiosulphates as possible impurities. It can therefore be concluded that sulphur fed as methionine is incorporated into seminal ergothioneine.

It can also be seen from Table 5 that sperm, and in particular, the ethanol-insoluble portion, acquire an appreciable radioactivity. This, however, reaches the maximum only 4–5 weeks after the administration of [^{35}S]methionine. With regard to blood it is interesting to note the high initial labelling of the blood plasma (561) in contrast to the blood cells (153). By the 4th week, however, the activity was the same in cells and plasma (Fig. 1).

Experiments with [^{35}S]*ergothioneine*

[^{35}S]Ergothioneine (0·4 g.) giving 2×10^7 counts/min. (calculated from the count at 'infinite thinness' using an end-window counter) was dissolved in 400 ml. water; 30 ml. of this solution was mixed with the food of boar *A* on 13 successive days, starting 4 months after the termination of the methionine experiment, by which time the activity of the semen due to methionine had reached a very low level.

Table 6. *Radioactivity of* $BaSO_4$ *prepared by the fractionation of the constituents of boar urine, blood and semen collected on the 5th and 9th days after feeding* [^{35}S]*ergothioneine*

(Values are expressed as counts/min. in 1 sq.cm. sample of 'infinite thickness'.)

Seminal plasma (ethanol soluble)	
Free + ethereal sulphate	79
Bromine-oxidizable S	695
Blood	
Plasma. Total S	6
Cells. Total S	32
Urine	
Total S	4
Free sulphate	2
Ethereal sulphate	2
Bromine-oxidizable S	2

Urine samples were collected on the 3rd and 5th days after feeding had been terminated, and semen was collected on the 5th and 9th days. The combined ejaculates represented 1 l. of semen and contained 124 mg. of ergothioneine. The bromine-oxidizable fraction from the seminal plasma was radioactive (Table 6). As both the urine samples had negligible

activity this could not have been due to urinary contamination. It is evident, therefore, that orally administered ergothioneine is incorporated into seminal plasma by the boar.

Examination of the blood showed higher labelling in the cells than in the plasma.

DISCUSSION

The demonstration that inorganic sulphate is unable to act as a source of sulphur for either blood or seminal ergothioneine is in accordance with observations on the metabolic inertness of sulphate in general. Sulphate is not utilized readily in the animal body except in certain cases such as in the formation of chondroitin sulphuric acid and ethereal sulphates (Dziewiatkowski, 1949). In this respect, animals with one stomach such as the pig, behave differently from ruminants. Because of the synthetic ability of the rumen micro-organisms, postulated by Rimington & Bekker (1932), ruminants are capable, for example, of efficiently utilizing inorganic sulphate for the formation of cystine and methionine (Block, Stekol & Loosli, 1951). Thiolhistidine, the corresponding amino-acid to the betaine ergothioneine, is shown by the present study to be completely inactive as a precursor of either blood or seminal ergothioneine in the pig. In this respect the pig does not differ from the rat where thiolhistidine was found by Heath (1953) to be equally inactive. These results, together with the previous observation of Mann & Leone (1953) on the complete absence of thiolhistidine, either free or bound, from both boar vesicular secretion and seminal-vesicle tissue, makes it highly improbable that thiolhistidine is involved in the normal biosynthesis of ergothioneine. Thus, the present study shows once again that the products of normal animal metabolism are not necessarily formed from chemically closely related substances.

Methionine has been shown to be capable of supplying the sulphur for the biosynthesis of ergothioneine. Our experiments establish for the first time that ergothioneine can be synthesized by an animal. Labelled ergothioneine, administered by mouth, appeared in the semen. Hence, absorption of ergothioneine from the alimentary tract and its passage in an unchanged state into the seminal plasma is possible.

It is of interest that the spermatozoa can acquire sulphur from labelled sulphate and methionine even though the maximum radioactivity appears in the ejaculated spermatozoa only some weeks after feeding. This slow rate, however, is not altogether unexpected in view of the long time required for the processes of spermatogenesis, sperm maturation and sperm transport as compared with the time required for the formation of seminal plasma in the male accessory glands. Both [^{35}S]sulphate and [^{35}S]thiolhistidine, when fed to the pig, are rapidly excreted in the urine, mostly in an unchanged state. Heath (1953), in experiments with rats, has already shown that [^{35}S]ergothioneine is excreted in the urine partly unchanged and partly as sulphate. The urinary excretion of ^{35}S after feeding methionine was prolonged, the greater part appearing as sulphate.

There is no evidence that either labelled sulphate or thiolhistidine can provide a source of sulphur for ergothioneine in blood. Feeding [^{35}S]ergothioneine raised the activity of the total sulphur in the blood cells, thus, as in the case of the rat (Heath, 1953), dietary ergothioneine is incorporated into the cellular fraction of pig blood.

SUMMARY

1. Boars have been fed with ^{35}S-labelled sulphate thiolhistidine, methionine and ergothioneine.

2. The distribution of ^{35}S has been studied in the blood, urine and semen.

3. It has been shown that neither sulphate nor thiolhistidine is utilized in the biosynthesis of ergothioneine.

4. The sulphur of methionine is incorporated into ergothioneine.

5. Dietary ergothioneine appears in the seminal plasma of the boar.

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The Structure of the Naturally Occurring Phosphoglycerides

1. EVIDENCE DERIVED FROM ALKALINE-HYDROLYSIS STUDIES

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The structures hitherto assigned to the lecithins and other naturally occurring phosphoglycerides have been largely based on the analysis of acid or alkaline hydrolysates. The presence in these hydrolysates of a mixture of α- and β-glycerophosphates was formerly taken as evidence for the occurrence of both an α- and β-series of phosphoglycerides in nature (Karrer & Salomon, 1926; Suzuki & Yokoyama, 1930; MacLachlan *et al.* 1942; Welch, 1945; Burmaster, 1946*c*).

Bailly (1939), however, had earlier found that interconversion of α- and β-glycerophosphates takes place when either substance is heated in acid solution, and Folch (1942*a*) noted that several phosphoglycerides from different sources always gave the same ratio of α- and β-glycerophosphate concentrations when subjected to acid hydrolysis under standard conditions. Burmaster (1946*c*), employing a fractionation procedure stated by Suzuki & Yokoyama (1930) to effect a separation of naturally occurring phosphoglycerides into an α- and β-series, found that on alkaline hydrolysis each of the supposedly pure α- and β-fractions yielded a mixture of glycerophosphates which always contained the same ratio of α- to β-isomers. More recently, Baer & Kates (1948) have demonstrated that both the acid and alkaline hydrolysis of synthetic L-α-glycerylphosphorylcholine is accompanied by migration of the phosphate group, resulting in the formation of a mixture of β-, D-α- and L-α-glycerophosphates. Finally, Baer & Kates (1950*a*) have shown that the acid or alkaline hydrolysis of a series of synthetic L-α-lecithins is accompanied by a similar phosphate migration. That a migration of the phosphate group likewise occurs when naturally occurring lecithins are hydrolysed by acid or alkali seems certain, thus accounting for the formation of both the α- and β-glycerophosphates reported in the literature. It is interesting to note that an analogous case of phosphate migration has been encountered in the formation of isomeric mononucleotides by chemical hydrolysis of the naturally occurring ribonucleic acids (Cohn, 1950*a*, *b*; Brown, Haynes & Todd, 1950; Brown & Todd, 1952*a*, *b*).

In the work described in this paper, we have determined the proportions of β-, D-α- and L-α-glycerophosphates formed on acid and alkaline hydrolysis of three partially purified naturally occurring lecithins and of a sample of ox-brain 'kephalin', and have compared them with the hydrolysis products of synthetic phosphoglycerides of known constitution. We are not aware of any previously published complete analyses of the glycerophosphates obtained on chemical hydrolysis of naturally occurring phosphoglycerides. The results show clearly that on alkaline hydrolysis the natural phosphoglycerides behave in the same way as synthetic L-α-lecithin, but quite differently from synthetic DL-α-lecithin, DL-α-kephalin or β-kephalin. A preliminary account of these results has already appeared (Maguire, 1952).

Further work is now in progress in which natural lecithins are being subjected to enzymic hydrolysis, and the results of these experiments will be reported in subsequent papers.

EXPERIMENTAL

Analytical methods

Total N was determined in the distillation apparatus of Markham (1942) after micro-Kjeldahl digestion, using the catalyst of Chibnall, Rees & Williams (1943). Choline was estimated as the reineckate on $Ba(OH)_2$ hydrolysates by the procedure of Glick (1944). Ethanolamine N + serine N was determined on acid hydrolysates by alkaline periodate oxidation in Conway microdiffusion units and measurement of the NH_3 liberated (Burmaster, 1946*b*). Total P was determined as orthophosphate, after ashing about 100 μg. phosphoglyceride with 0·4 ml. 72% $HClO_4$, by the method of Berenblum & Chain (1938), using the micromodification described by Long (1943). Ammonium dihydrogen phosphate of A.R. purity was used as standard for both N and P determinations.

Materials

Phosphoglycerides from natural sources. A sample of ox-heart lecithin and several specimens of ovolecithin were isolated by the method of Pangborn (1951).

Soyabean lecithin was prepared from an acetone-insoluble fraction of soyabean lipids (Bibby and Co. Ltd., Liverpool), using the following procedure. The material (25 g.) was shaken with ethanol (500 ml.) at room temperature for 6 hr. After filtering off insoluble material, the extract was treated with 50% (w/v) $CdCl_2$ solution until precipitation was complete. Subsequent purification was carried out according to Pangborn (1951). Yield, 0·6 g.

Ox-brain 'kephalin' was prepared by the method of Folch (1942*b*). After removing the 'inositol phosphatide', the product obtained corresponded with 'fractions III–V', i.e. a mixture in which phosphatidylethanolamine and phosphatidylserine predominate.

All lecithin samples were dissolved in ethanol; the ox-brain 'kephalin' was dissolved in $CHCl_3$. These solutions (approx. 30 mg. dry wt./ml.) were stored in glass-stoppered flasks at 0°.

Analytical figures relating to these natural phosphoglycerides are given in Table 1. In spite of the unsatisfactory analysis shown by the soyabean lecithin, studies with this substance have been included since it behaved like the other lecithins on acid and alkaline hydrolysis.

Table 1. *Analysis of natural phosphoglycerides*

	Total N (%)	Choline N (%)	Hydroxy-amino N (%)	Total P (%)
Ovolecithin	1·86	1·72	0·04	3·94
Ox-heart lecithin	1·89	1·72	0·06	3·93
Soyabean lecithin	0·80	0·27	—	4·25
Ox-brain kephalin	1·53	0·12	0·99	3·41

Synthetic phosphoglycerides. (For consistency of nomenclature, the designations α and β refer to the position of attachment of the phosphate group to the glycerol molecule.) The following samples of synthetic phosphoglycerides were used: dipalmitoyl DL-α-lecithin, dimyristoyl DL-α-kephalin, dimyristoyl β-kephalin (Malkin & Bevan, 1951) and dimyristoyl L-α-lecithin (Baer & Kates, 1950*b*). The term 'kephalin' is generally applied to a mixture of naturally occurring substances, containing both phosphatidylethanolamine and phosphatidylserine, but the synthetic kephalins used in this work were the ethanolamine derivatives only.

Barium α-glycerophosphates. Barium L-α-glycerophosphate (L-α-GPA) (Baer & Fischer, 1939) and barium DL-α-glycerophosphate (DL-α-GPA) (King & Pyman, 1914) were used as standards.

L-α-Glycerophosphate dehydrogenase. An active enzyme, obtained from fresh sheep brain by the method of Weil-Malherbe (1937), was dispersed in 0·1 M-sodium veronal-acetate buffer, pH 8·0 (Michaelis, 1931), and stored at 0°. There was no loss of activity during 3–4 weeks.

Hydrolysis of phosphoglycerides and isolation of mixed glycerophosphates

Into a series of Pyrex tubes (19 × 150 mm.), containing a solution of the phosphoglyceride (80–90 mg. dry wt.), were pipetted 2 ml. of either N-NaOH, saturated aqueous $Ba(OH)_2$ or N-HCl. Lecithin samples were always first dissolved in 0·5 ml. ethanol, while the 'kephalins' were dissolved in 0·5 ml. $CHCl_3$. Closely fitting cold fingers were used as condensers. The tubes were then placed in an oil bath at 110–120°. Heating was continued for 2, 3 and 7 hr. using NaOH, $Ba(OH)_2$ and HCl hydrolysis, respectively. This is essentially the procedure of Baer & Kates (1950*a*) on a reduced scale.

At the end of the appropriate time, solvent was removed by withdrawing the cold finger. Each hydrolysate was then treated with distilled water (5 ml.) and neutralized with HCl or NaOH. Free fatty acids were precipitated by addition of 1 ml. 15% (w/v) trichloroacetic acid (TCA); after standing at room temperature for 15 min., they were filtered off and the filtrate collected in a 60 ml. centrifuge tube. The precipitate was washed twice with 2 ml. portions of 2·5% TCA. The combined filtrate and washings were treated with 2 N-barium acetate (0·5 ml.) and brought to pH 8·4 (phenolphthalein) with 10 N-NaOH. Ethanol (3 vol.) was then added and after thorough mixing, the stoppered tube was chilled overnight. The precipitate of barium glycerophosphates was spun down and the supernatant discarded. The precipitate was redissolved in 3 ml. water with the addition of the minimum amount of HCl. The solution was then transferred to a 15 ml. graduated centrifuge tube, the pH adjusted to 8·4 and the barium glycerophosphate reprecipitated with 3 vol. ethanol. After chilling for 2–3 hr. the precipitate was spun down and dried *in vacuo* over H_2SO_4. The dry solid was then dissolved in 1 ml. 0·01 N-HCl and the solution stored at 0° until required for analysis. The barium glycerophosphates isolated in this way contained not less than about 85% of the original phosphoglyceride P.

Determination of total glycerophosphate

Total glycerophosphate (β, D-α and L-α) was determined by a micromodification of the method of Burmaster (1946*a*). A sample of the barium glycerophosphate solution (equivalent to about 3·5 μg. P) was treated with 1·4 M-$HClO_4$ (1 ml.) followed by 0·01 M-KIO_4 (0·2 ml.), and the mixture placed in a boiling-water bath for 1 hr. A water blank and inorganic phosphate standard were similarly treated. Inorganic phosphate estimations were carried out on the cooled solutions. The method gave 100% of the theoretical values, based on total P content, when tested with the two samples of synthetic barium α-glycerophosphate and with a commercial preparation of sodium β-glycerophosphate.

Determination of α-glycerophosphate

The amount of total α-glycerophosphate (D-α and L-α) was determined by a periodate oxidation procedure based on the method of Fleury & Lange (1933), in which α-glycerophosphate is rapidly and quantitatively oxidized by one equivalent of HIO_4 at room temperature, while β-glycerophosphate remains unattacked.

The barium glycerophosphate solution (0·1 ml., equivalent to 100–250 μg. α-glycerophosphate P), was treated with 1 ml. approx. 0·02 N-KIO_4 and the mixture, adjusted to pH 4, allowed to stand for 1 hr. To the solution was then added 2 drops of M-Na_2SO_4 (to precipitate Ba) followed by 2 drops of M-$NaHCO_3$, to raise the pH into the range suitable for the selective titration of the excess KIO_4 in the presence of KIO_3. Solid KI (about 0·1 g.) was then added and the liberated iodine titrated with a solution of standard 0·02 N-sodium arsenite in 0·1 M-phosphate buffer, pH 7·0 (Washburn, 1908), using 2 drops 1% starch solution as indicator. A blank titration on 1 ml. of the KIO_4 solution was carried out at the same time, the difference between this and the experimental value being used to calculate the α-glycerophosphate content of the solution. This method gave theoretical results with the samples of synthetic barium L-α- and DL-α-glycerophosphates.

Determination of L-α-glycerophosphate

The amount of L-α-glycerophosphate contained in a phosphoglyceride hydrolysate was determined manometrically by measuring the O_2 uptake in the presence of

L-α-glycerophosphate dehydrogenase with pyocyanine (Weil-Malherbe, 1937) as O_2 carrier. Conventional Warburg technique was employed.

The main chamber of the Warburg vessel contained 2·0 ml. buffered enzyme dispersion, pH 8·0, and 0·5 ml. 0·02M-pyocyanine, a filter-paper roll soaked in 2N-KOH (0·2 ml.) being placed in the centre well. Into the side bulb was pipetted 0·2–0·4 ml. barium glycerophosphate solution (equivalent to approx. 150 μg. of L-α-glycerophosphate P) and 0·1 ml. 0·01M-phenol red. This mixture was then titrated with 0·1N-NaOH until the indicator turned a definite pink colour (pH about 8·0); distilled water was added to bring the total fluid volume in the side bulb to 0·7 ml. After 15 min. temperature equilibration at 38°, the contents of the side bulb were tipped into the main chamber and readings taken until no further O_2 uptake occurred; the reaction was usually completed within 45 min. The final reading was corrected for the small blank O_2 uptake of the enzyme without added substrate.

Theoretical O_2 uptakes were obtained with known amounts of DL-α- and L-α-glycerophosphates.

RESULTS

Acid hydrolysis

When an acidified solution of sodium β-glycerophosphate is heated to boiling for 1 hr., migration of the phosphate group occurs and equilibrium is reached when about 85% has been converted into DL-α-glycerophosphate (Bailly, 1939). It follows, therefore, that acid hydrolysis of all phosphoglycerides, whether they be the synthetic β-, L-α- or DL-α-compounds or the naturally occurring substances will also give an equilibrium mixture of about 15% β-glycerophosphate and 85% DL-α-glycerophosphate. Therefore, analysis of the products of acid hydrolysis can give no information regarding the configuration of the phosphoglycerides. In spite of this fact, we have found it valuable to subject all our samples of naturally occurring phosphoglycerides to acid hydrolysis as a routine, for any deviation from the expected relative proportions of the glycerophosphates would indicate either the presence of some interfering impurity in the materials used or a failure of the analytical procedure. Thus, when a sample of crude ox-brain 'kephalin', prepared by the method of Folch (1942*b*), was hydrolysed in N-HCl under the conditions already defined, the results obtained were not those to be expected for a simple phosphoglyceride. The discrepancy was found to be due to the presence of 'inositol phosphatide', which on acid hydrolysis yields a water-soluble inositol phosphate, forming an alcohol-precipitable barium salt, which in turn reacts with potassium periodate at room temperature. The net result was an erroneously high value for the apparent α-glycerophosphate; indeed, the observed value was appreciably higher than that found for total glycerophosphate. When the 'inositol phosphatide' was removed by ethanol precipitation, however, to give Folch's 'fractions III–V', normal values were found after acid hydrolysis. All the naturally occurring phosphoglycerides used in the present work were ethanol-soluble, so that there was no possibility of interference from contaminating 'inositol phosphatide'. Results of the acid-hydrolysis studies are shown in Table 2. It will be noted that the α-glycerophosphate is always present in the racemic form.

Table 2. *Composition of glycerophosphates formed on acid hydrolysis of synthetic and naturally occurring phosphoglycerides*

(Acid hydrolysis carried out by refluxing with N-HCl at 110–120° for 7 hr. Determination of total, α- and L-α-glycerophosphates, as given in Experimental section. Values are given as percentage of total glycerophosphate.)

	Analysis of glycerophosphates			
	β	α	L-α	D-α
(*a*) Synthetic phosphoglycerides				
Dimyristoyl L-α-lecithin	10	90	46	44
Dipalmitoyl DL-α-lecithin	14	86	44	42
Dimyristoyl DL-α-kephalin	12	88	43	45
Dimyristoyl β-kephalin	12	88	43	45
(*b*) Naturally occurring phosphoglycerides				
Ovolecithin	11	89	46	43
Ox-heart lecithin	13	87	44	43
Soyabean lecithin	18	82	39	43
Ox-brain kephalin	15	85	42	43

Alkaline hydrolysis

The phosphate group does not migrate when α- or β-glycerophosphates are heated in alkaline solution (Verkade, Stoppelenburg & Cohen, 1940). Partial isomerization does occur, however, when synthetic L-α-glycerylphosphorylcholine (Baer & Kates, 1948) or L-α-lecithins (Baer & Kates, 1950*a*) are subjected to alkaline hydrolysis. The α-glycerophosphate is, however, almost entirely in the L form. It has been found in the present work that partial isomerization also takes place when synthetic DL-α-lecithin, DL-α-kephalin and β-kephalin are hydrolysed with alkali, and that the composition of the glycerophosphate mixture differs from that formed from L-α-lecithin in that the α-glycerophosphate is racemic. The naturally occurring phosphoglycerides on alkaline hydrolysis all yield glycerophosphate mixtures similar to that found with L-α-lecithin and differing from that found with β- and DL-α-phosphoglycerides. These results are summarized in Table 3.

DISCUSSION

The only experimental procedure used in this work which requires comment is the use of the L-α-glycerophosphate dehydrogenase for the specific

Table 3. *Composition of glycerophosphates formed on alkaline hydrolysis of synthetic and naturally occurring phosphoglycerides*

(Alkaline hydrolysis carried out by refluxing with N-NaOH for 2 hr. or sat. $Ba(OH)_2$ for 3 hr., at 110–120°. Determination of total, α- and L-α-glycerophosphates, as given in Experimental section. Values are given as percentage of total glycerophosphate.)

	Analysis of glycerophosphates after hydrolysis with NaOH				Ba(OH)$_2$			
	β	α	L-α	D-α	β	α	L-α	D-α
(a) Synthetic phosphoglycerides								
Dimyristoyl L-α-lecithin	51	49	43	6	56	44	39	5
Dipalmitoyl DL-α-lecithin	53	47	23	24	52	48	24	24
Dimyristoyl DL-α-kephalin	50	50	26	24	50	50	26	24
Dimyristoyl β-kephalin	52	48	22	26	55	45	23	22
(b) Naturally occurring phosphoglycerides								
Ovolecithin	53	47	41	6	54	46	42	4
Ox-heart lecithin	52	48	42	6	54	46	40	6
Soyabean lecithin	53	47	42	5	54	46	42	4
Ox-brain kephalin	51	49	47	2	53	47	42	5

determination of L-α-glycerophosphate, present in the phosphoglyceride hydrolysates. This method was necessarily adopted because polarimetric analysis, used successfully by Baer & Kates (1950*a*) on hydrolysed samples of synthetic lecithins, appeared unsuitable for the determination of small amounts of L-α-glycerophosphoric acid of specific optical rotation $-1{\cdot}2°$, where the presence of traces of optically active impurities would render the method useless. The validity of the enzymic method has been confirmed by the fact that theoretical values were obtained with pure DL-α- and L-α-glycerophosphates and moreover the values obtained in this way on hydrolysates of a synthetic L-α-lecithin were identical with those published by Baer & Kates (1950*a*) using their polarimetric method.

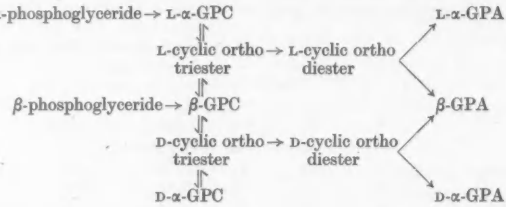
The main result which emerges from this study is that on alkaline hydrolysis the naturally occurring phosphoglycerides and synthetic dimyristoyl L-α-lecithin give exactly the same proportions, of β-, L-α- and D-α-glycerophosphates. Since, on the other hand, dipalmitoyl DL-α-lecithin, dimyristoyl DL-α-kephalin and dimyristoyl β-kephalin give different proportions of the L-α- and D-α-glycerophosphates, it seems reasonable to infer that the naturally occurring phosphoglycerides belong to the L-α-series. This conclusion is consistent with the observation that the so-called 'hydrolecithin', i.e. dipalmitoyl lecithin, first isolated from the larvae of the tapeworm *Cysticercus fasciolaris* (Lesuk & Anderson, 1941) and later from several ox tissues (Thannhauser, Benotti & Boncoddo, 1946; Thannhauser & Boncoddo, 1948) appears to be identical with synthetic dipalmitoyl L-α-lecithin (Baer & Kates, 1950*b*). Furthermore, the glycerylphosphorylcholine, isolated by Schmidt, Hershman & Thannhauser (1945) from autolysed pancreas and assumed to have arisen by the action of lecithinases A and B on the endogenous lecithin, was likewise stated to possess the L-α-configuration. It is also interesting to note that the naturally occurring 'acetal phosphatide' of ox brain, on catalytic removal of the fatty aldehyde group with aqueous mercuric chloride, gives only L-α-glycerylphosphorylethanolamine, showing that this phosphoglyceride also belongs to the L-α-series (Thannhauser, Boncoddo & Schmidt, 1951).

Finally, it seems desirable to consider briefly the mechanism by which the various isomeric glycerophosphates arise. Baer & Kates (1950*a*) have shown that when a synthetic L-α-lecithin is subjected to alkaline hydrolysis, the fatty acids are liberated much more rapidly than is the free choline, an observation which clearly indicates that the main intermediate product is L-α-glycerylphosphorylcholine (L-α-GPC). Presumably, synthetic β-phosphoglycerides will likewise yield β-glycerophosphorylcholine (β-GPC) as intermediate product.

Two theories have been proposed to explain the mechanism of alkaline hydrolysis of phosphate diesters, such as the GPC's, which contain an adjacent hydroxyl group. In the scheme of Baer & Kates (1950*a*), L-α-GPC is converted via the L-cyclic ortho triester into β-GPC, which in turn may give rise to both D-α- and L-α-GPC by way of the respective D- and L-cyclic ortho triesters. These three isomeric GPC's are then hydrolysed to the corresponding glycerophosphates. The theory does not imply, of course, that an equilibrium mixture of the three GPC's is formed before any choline is split off; the removal of choline may proceed simultaneously with the isomerization reactions. This theory, however, seems unable to account for the different ratios of β-GPA to D-α-GPA which have been found in the alkaline hydrolysates of synthetic β- and L-α-phosphoglycerides in the present work. Since it

is postulated that β-GPA and D-α-GPA can only arise through the intermediate formation of β-GPC, then the same ratio would be expected independently of whether the β-GPC originates from a β- or L-α-phosphoglyceride.

Brown & Todd (1952*b*) have suggested that loss of choline is more likely to take place from the cyclic ortho triesters than from the open chain GPC's. In the case of L-α-GPC hydrolysis, conversion to the L-cyclic ortho triester, loss of choline to give a cyclic diester and subsequent ring opening would yield a mixture of β-GPA and L-α-GPA. If it is assumed that the cyclic diesters have about equal chances of splitting at either of the two ester bonds, then approximately equal amounts of β-GPA and L-α-GPA will be formed. The conversion of some of the L-cyclic ortho triester by way of β-GPC to the D-cyclic ortho triester and degradation of the latter to β-GPA and D-α-GPA would readily account for the small quantity of D-α-GPA found in alkaline hydrolysates of L-α-GPC. In the alkaline hydrolysis of β-GPC, on the other hand, there will be formed 50 % each of the L- and D-cyclic ortho triesters. Making the same assumption as before, the former will give rise to 25 % each of β-GPA and L-α-GPA, while the latter will yield 25 % each of β-GPA and D-α-GPA. It will be seen that these calculations for the breakdown of β-GPC and L-α-GPC are consistent with the results obtained for the alkaline hydrolysis of β- and L-α-phosphoglycerides, shown in Table 3. By the same reasoning it may be shown that β- and DL-α-phosphoglycerides should give the same mixture of glycerophosphates on alkaline hydrolysis, as has been found by experiment. This mechanism of hydrolysis can be represented as follows:



The experimental results obtained in the present work may therefore be regarded as providing support for the validity of the theory of Brown & Todd (1952*b*).

SUMMARY

1. The amount of L-α-glycerophosphate, in an acid or alkaline hydrolysate of a phosphoglyceride, has been determined manometrically, using the L-α-glycerophosphate dehydrogenase of sheep brain.

2. Alkaline hydrolysis, using either N-sodium hydroxide or saturated aqueous barium hydroxide, of four naturally occurring phosphoglycerides, yields proportions of β-, L-α- and D-α-glycerophosphates identical with those given by synthetic dimyristoyl L-α-lecithin and quite distinct from those obtained on alkaline hydrolysis of synthetic β- or DL-α-phosphoglycerides. This observation indicates that the naturally occurring phosphoglycerides belong to the L-α-series.

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Animal Fats

1. THE COMPONENT ACIDS OF DEER FAT AND OF CAMEL FAT

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Study of the detailed composition of seed fats has led to the realization that there is a close relationship between the classification of the parent plants on the basis of fat composition and that arrived at by botanists on more general grounds. Among land animals, however, only very tentative generalizations can be made because of the paucity of detailed analyses of animal fats. Hilditch (1947, p. 85) has drawn attention to this deficiency and it has been further emphasized by Barker & Hilditch (1950) who list the land animals, the fats of which have been satisfactorily analysed. Further analyses have since been reported for the fats obtained from hippopotamus (Barker & Hilditch, 1950), badger (Gupta, Hilditch & Meara, 1950), horse (Brooker & Shorland, 1950; Gupta & Hilditch, 1951), rabbit (Clément & Meara, 1951) and tiger (Pathak & Agarwal, 1952).

We are interested in this problem, and over the last few years have collected, from fifteen different animals, samples of fats which we hope to study. These vary in weight from 20 to 600 g. and cover an iodine-value range from 26 to 108. The fats from many of these animals have not previously been examined in any detail, and even in the few cases where satisfactory analyses have been reported it is considered worth while to repeat the work in order to study variation between different members of the same species. In the present paper we record our analyses of deer and of camel fat; these relatively simple examples were purposely selected as a convenient beginning to this work in order to gain experience for the study of more complicated fats. The methods of analysis may have to be varied for different fats. Modifications have already been made in the light of experience and the methods reported in this paper are, therefore, not in every detail the most satisfactory ones. Improvements will be reported later as they are applied.

Reports on the composition of deer fat seem to be limited to the recording of physical data and characteristics (largely summarized by Lewkowitsch, 1922) and to the work of Baughman, Jamieson & McKinney (1929) and of Treadwell & Eckstein (1939). The former authors studied five samples of American reindeer fat of iodine value 33·7–39·4. Treadwell & Eckstein have reported the saponification value, iodine value and thiocyanogen value of fats obtained from various parts of five Virginia white-tailed deer (*Odocoileus virginianus borealis*). Apart from the fat isolated from the mammary gland there is little variation in these samples. These earlier results are summarized in Table 8.

Previous recorded data on camel fat appear to be limited to a report by Armstrong & Allan (1924), in which only the major component acids, palmitic (37 %), stearic (16%) and oleic (47%) are recognized.

EXPERIMENTAL

Preliminary work

In planning this series of analyses, it was anticipated that some of the fatty-acid fractions to be examined would be rather smaller than usual, either on account of the relatively small amount of material available or because of the concentration of minor components in a small fraction. These fractions (6–12 g.) have been distilled through a Dixon 6 in.

still (Dixon, 1945), and the resulting fractions (0·7–1·0 g.) examined in the usual way. In devising a method of determining the saponification equivalent on about 0·6 g. of ester we were unable to get satisfactory results by the double indicator procedure of Marcali & Rieman (1946), and eventually found that the standard method on a reduced scale gave the best results. The ester was hydrolysed with 0·5 N-ethanolic KOH (10 ml.) for 30 min. and the excess alkali titrated against 0·5 N-HCl using a 5 ml. microburette. Table 1 gives the results of analysing various quantities of a mixture of crude myristic, palmitic and stearic acids. In Exp. *A*, the Towers (T. 117) column was used and equivalents determined by the standard procedure; in Exps. *B* and *C*, the Dixon 6 in. still was used and the equivalents were determined by the above method. The main difficulty lies in the satisfactory examination of the residue, the proportion of which tends to increase as the amount of material distilled decreases. These results are considered to show a fair agreement, particularly between the major components.

Table 1. *Results obtained by distilling various quantities of the same mixture*

Exp.	*A*	*B*	*C*
Amount distilled (g., approx.)	40	12	6
No. of distilled fractions	12	11	7
Calculated composition (%)			
Lauric	0·6	0·3	—
Myristic	10·2	10·0	10·1
Palmitic	40·1	39·0	39·0
Stearic	38·7	39·5	36·6
Arachidic	—	0·6	3·7
Tetradecenoic	0·4	0·4	0·5
Octadecenoic	10·0	10·2	10·1

As we have all the necessary facilities for doing low-temperature crystallizations, we decided to carry out all our separations by this method and to dispense with the lead salt separation, thus following the methods used by Brooker & Shorland (1950), Gupta & Hilditch (1951), and Clément & Meara (1951). It is, nevertheless, desirable that a method of crystallization be used which separates saturated and unsaturated acids as effectively as does the lead salt procedure. The former authors have used ether or acetone as solvents at −30° to −35° to do this, but as we were able to use a refrigerator maintained at −20° we tried to find a solvent which would separate saturated and unsaturated acids at this temperature. Preliminary results showed that equilibrium is attained in less than 18 hr. and that there is no advantage in using solutions more dilute than 10 ml. of solvent/g. of acids. Table 2 shows the results obtained on separating mixed tallow acids with various solvents at this dilution at −20° overnight. The results of a lead salt separation are included for comparison. Table 3 contains similar results obtained with a mixture of crude palmitic (12%), stearic (13%) and oleic (75%) acids. Perusal of Table 2 shows that ethyl acetate, light petroleum or methanol give results comparable with those obtained in the lead salt separation and that even better results follow the use of methanol containing up to 5% of water. Similar satisfactory results were obtained with these solvents on the mixture containing only about 25% of saturated acids, though in this case aqueous methanol (5%) gave less satisfactory results.

Table 2. *Low-temperature crystallization of tallow acids*

(Iodine value 42·8; about 10 g.)

	Insoluble		Soluble	
Solvent	Wt. (%)	Iodine value	Wt. (%)	Iodine value
Lead salt separation	52·8	5·6	47·2	83·6
Acetone	46·1	2·7	53·9	75·5
Ether	35·1	2·8	64·9	62·3
Ethyl acetate	48·9	2·6	51·1	82·0
Light petroleum	51·7	6·0	48·3	80·9
Methanol	49·7	3·5	50·3	79·8
Methanol-water (1%)	49·2	3·4	50·8	74·3
Methanol-water (2%)	50·2	3·5	49·8	82·4
Methanol-water (5%)	52·3	3·9	47·7	85·8
Methanol-water (10%)	57·5	10·1	42·5	87·8
Methanol-water (15%)	55·1	6·9	44·9	86·4
Dichloromethane	47·8	2·0	52·2	79·3
Chloroform, Carbon tetrachloride	Results so obviously inferior that the two fractions were not examined.			

Table 3. *Low-temperature crystallizations of mixed acids*

(Approx. 10 g. mixed acids, containing about 25% saturated acids.)

	Insoluble		Soluble	
Solvent	Wt. (%)	Iodine value	Wt. (%)	Iodine value
Light petroleum	24·9	12·3	75·1	93·8
Ethyl acetate	24·0	7·7	76·0	93·5
Methanol	25·5	8·8	74·5	95·2
Methanol-water (5%)	29·1	15·8	70·9	96·6

It was finally decided to use methanol as crystallizing solvent, the above data having shown that a small amount of water in this solvent does not significantly affect the results.

Source and preparation of fat

The deer fat (230 g.), obtained from a butcher in Perth, had come from a stag (*Cervus elaphus*) reared in Scotland. The crude material, which was very hard after being kept at

Table 4. *Characteristics of deer fat and camel fat*

	Fat			Mixed acids			
	Iodine value	Saponification equivalent	F.F.A.*	Iodine value	Saponification equivalent	$E^{1\%}_{1\,cm.}$ 234 mμ.†	$E^{1\%}_{1\,cm.}$ 268 mμ.†
Deer	35·5	284·9	2·0	32·4	266·2	20·3	6·9
Camel	35·1	282·2	2·1	36·8	270·4	22·2	4·6

* Free fatty acid as % oleic.

† Ultraviolet absorption after alkali isomerization (Hilditch, Morton & Riley, 1945; Hilditch, Patel & Riley, 1951).

0°, was crushed and extracted with light petroleum; removal of the solvent left 151 g. of fat, the characteristics of which are summarized in Table 4.

The camel fat was extracted from material kindly supplied by Mr G. T. Iles, Zoological Superintendent, Belle Vue, Manchester. The animal was a male bactrian camel (*Camelus bactrianus*) aged about 7 years and the fat was taken from the mesentery. Its diet consisted of meadow and clover hays supplemented by a daily feed of chopped vegetables and fruit along with chopped hay, oats, bran and horse-feed cubes mixed together with a little cod-liver oil; fresh grass was also available. The material supplied was autoclaved to render it sterile and then crushed and extracted with acetone. The characteristics of the fat are given in Table 4.

Method of analysis

In view of the relatively high proportion of saturated acids expected in these fats, the mixed acids were crystallized first from methanol (10 ml./g.) at −20° and the soluble acids were subsequently crystallized from acetone (10 ml./g.) at −50 to −55°. This gave three fractions (*A*, *B* and *C* in order of increasing solubility) details of which are summarized in Table 5. Each fraction was subsequently methylated and distilled, the iodine value and saponification equivalent of each fraction being determined. The composition of each fraction and hence of the fat has been calculated by the methods described by Hilditch (1947, pp. 484–512) and is given in Tables 6 and 7. Spectroscopic measurements were made after isomerization of the acid fractions only; better results are obtained if selected ester fractions are used and this procedure is followed in all subsequent work. In these two fats, however, the amount of polyethenoid acids, other than C_{18}, is negligible in the case of deer fat and very small in the camel fat so that the error introduced in this way is hardly significant. Absorption measurements on unisomerized acids gave very small values (probably due to oxidation) which have been neglected.

Table 5. *Low-temperature crystallization of deer and camel fatty acids*

	Deer			Camel		
Fraction	*A*	*B*	*C*	*A*	*B*	*C*
Weight (g.)	61·18	20·73	15·97	38·70	21·94	8·43
Percentage of total	62·5	21·2	16·3	56·0	31·8	12·2
Iodine value	5·9	67·3	122·7	2·9	69·0	107·5
Absorption after isomerization						
$E^{1\%}_{1\,cm.}$ at 234 mμ. (180°/60 min.)	—	16·6	209·2	—	12·4	148·7
$E^{1\%}_{1\,cm.}$ at 268 mμ. (170°/15 min.)	—	2·0	80·9	—	1·5	37·5

Table 6. *Component acids of deer fat*

	Fraction					
	A	*B*	*C*	Total	% (wt.)*	% (mol.)*
Lauric	—	—	0·01	0·01	—	—
Myristic	1·09	2·26	1·08	4·43	4·4	5·3
Palmitic	22·32	2·42	0·32	25·06	25·1	26·7
Stearic	33·96	0·85	0·42	35·23	35·4	33·9
Arachidic	1·34	0·16	—	1·50	1·5	1·3
Tetradecenoic	—	0·09	0·40	0·49	0·5	0·6
Hexadecenoic	0·26	0·89	1·65	2·80	2·8	3·0
Octadecenoic	3·44	13·99	7·72	25·15	25·2	24·3
Octadecadienoic	—	0·34	2·26	2·60	2·6	2·5
Octadecatrienoic	—	0·08	2·38	2·46	2·5	2·4
Unsaponifiable	0·09	0·12	0·06	0·27	—	—

* Excluding unsaponifiable material.

Table 7. *Component acids of camel fat*

	Fraction					
	A	*B*	*C*	Total	% (wt.)*	% (mol.)*
Myristic	1·24	3·93	1·08	6·25	6·3	7·4
Palmitic	25·67	2·63	0·39	28·69	28·8	30·5
Stearic	25·81	1·51	0·04	27·36	27·4	26·2
Arachidic	1·45	0·09	—	1·54	1·6	1·3
Tetradecenoic	—	0·18	0·36	0·54	0·5	0·6
Hexadecenoic	0·14	1·22	1·83	3·19	3·2	3·4
Octadecenoic	1·55	20·08	4·67	26·30	26·4	25·3
Octadecadienoic	—	0·38	1·48	1·86	1·9	1·8
Octadecatrienoic	—	0·09	0·83	0·92	0·9	0·9
As eicosenoic†	0·17	1·57	1·27	3·01	3·0	2·6
Unsaponifiable	—	0·08	0·26	0·34	—	—

* Excluding unsaponifiable material.

† Includes all unsaturated acids higher than C_{18}. (Average unsaturation about −3H.)

Table 8. *Component acids of some 'stearic-rich' animal fats*

(Values quoted are % by wt.)

	Deer			Camel		Sheep	Ox	Pig	Hippopotamus
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*j*)
Myristic	4·4	7	65–70	6·3	—	2–4	3·0	1·0*	2·3
Palmitic	25·1	35		28·8	37	23–28	29·2	28–30	27·1
Stearic	35·4	20		27·4	16	15–31	21·0	12–16	22·2
Arachidic	1·5	1		1·6	—	—	0·4	—	1·1
Tetradecenoic	0·5	—	—	0·5	—	—	0·6	0·2*	0·4
Hexadecenoic	2·8	—	—	3·2	—	1·0–2·5	2·7	2·5*	2·2
Octadecenoic	25·2	37	27–31	26·4	47	36–46	41·1	41–48	39·3
Octadecadienoic	2·6	—	3–5	1·9	—	4–6	1·8	6–7	3·5
Octadecatrienoic	2·5	—	—	0·9	—	—	—	—	1·5
C_{20-22} unsaturated acids	—	—	—	3·0	—	0·5*	0·2	2·0*	0·4

(*a*) and (*d*), present work. (*b*), Baughman, Jamieson & McKinney (1929). (*c*), Treadwell & Eckstein (1939). (*e*), Armstrong & Allan (1924). (*f*), (*g*) and (*h*), quoted by Hilditch (1947, p. 105). (*j*), Barker & Hilditch (1950).
* Approximate values.

Identification of acids

The results obtained by these methods have been qualitatively confirmed by the isolation of palmitic and stearic acids from appropriate fractions and by the preparation of derivatives of some of the unsaturated acids. For both fats, oleic, linoleic and linolenic acids were shown to be present by the isolation of the usual dihydroxy-, tetrabromo-, and hexabromo-stearic acids respectively. In all cases satisfactory melting points and mixed melting points with authentic specimens were recorded. Suitable fractions from the analysis of camel fat also gave myristic acid and dihydroxypalmitic acid (m.p. 123–124·5°; literature, 125°) as a derivative of hexadec-9-enoic acid. When a deer-fat fraction rich in hexadecenoic acid was similarly oxidized by the method of Lapworth & Mottram (1925), the hydroxy acid obtained analysed satisfactorily for dihydroxypalmitic acid (Found: C, 66·4; H, 11·4. Calc. for $C_{16}H_{32}O_4$: C, 66·6; H, 11·2%) but melted sharply at 116°. The quantity available did not permit further investigation of this interesting point but the hexadecenoic acid present in deer fat may be an isomer of the more common hexadec-9-enoic acid. Hilditch & Lovern (1928) have previously reported a dihydroxypalmitic acid, m.p. 115°, from the hexadecenoic acid present in sperm oil.

RESULTS AND DISCUSSION

The results obtained in the present work, along with some other relevant data, are summarized in Table 8. Comparing the present analyses with previous results for the same species it will be noticed that our results agree fairly well with the less detailed analyses of the Virginia white-tailed deer but less well with that of the American reindeer fat. Agreement between the figures for camel fat is only rough. These differences are not surprising in view of the approximate methods used in the earlier work.

Both deer and camel fats are more saturated than the average sheep or ox tallow, and this is reflected mainly in the increased content of stearic acid and decreased amount of oleic acid. In deer fat particularly the stearic figure is exceptionally high, whilst in both cases the amount of oleic acid is remarkably low. In general, the results are those to be expected of 'stearic-rich animal depot fats'; the content of palmitic acid (26·7 and 30·5% (mol.), respectively) lies practically within the accepted range of 30 ± 3% (Banks & Hilditch, 1931; Hilditch & Longenecker, 1937); the remainder of the acids are almost entirely C_{18}; and smaller proportions of myristic, arachidic, tetradecenoic and hexadecenoic acids are also present. Deer fat does not contain any unsaturated acids higher than C_{18}, but the present sample of camel fat contains 3% of higher unsaturated acids; these may originate in the cod-liver oil supplied in the camel's diet. In spite of the fact that the amount of oleic acid is lower than usual, both fats contain di- and tri-ethenoid acids of the C_{18} series. These appear to be largely, if not entirely, the normal linoleic and linolenic acids present in seed fats.

SUMMARY

1. A systematic study of animal fats has been begun and the composition of deer fat and of camel fat are reported.

2. Satisfactory methods of studying smaller quantities of ester than is usual in this work are described.

3. Crystallization from methanol at $-20°$ is an effective method of separating saturated and unsaturated acids.

4. Both deer and camel fat are 'stearic-rich' with palmitic (25 and 29%), stearic (35 and 27%) and oleic acids (25 and 26%) as major components, and myristic, arachidic, tetradecenoic, hexadecenoic, octadecadienoic and octadecatrienoic acids as minor components. Camel fat also contains some higher unsaturated acids.

We wish to thank Mr G. T. Iles of Belle Vue, Manchester, for supplying us with the sample of camel fat used in this investigation, the University of Glasgow for a scholarship held by one of us (R. P. P.) and Imperial Chemical Industries Ltd. for a grant towards the purchase of apparatus.

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Animal Fats

2. THE COMPONENT ACIDS OF PYTHON FAT

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The need for more information on the composition of animal fats has been emphasized in the introduction to this series (Gunstone & Paton, 1953), and we now report the composition of python fat.

Previous reference to python fat is limited to an account by Tsujimoto & Kobayaschi (1920) of a sample of python oil of acid value 0·6, saponification value 194·1, and iodine value 80·3, which yielded some ether-insoluble polybromides (2·1%) indicating the presence of highly unsaturated acids, probably of the C_{20} and C_{22} series. The characteristics of some other snake oils have been given by Pollard & McLaughlin (1950), but no analyses are reported. A few detailed analyses of the depot fats of Amphibia and Reptilia have been published, and from these few results some tentative generalizations have been drawn. More information is clearly needed.

EXPERIMENTAL

Source and preparation of the fat

The sample of python fat, kindly supplied by Mr G. T. Iles of Belle Vue, Manchester, had been obtained from a female reticulated python (*Python reticulatus*), 19 ft. long and 21 in. around its greatest girth. It had been fed on goats, ducks, hens and rabbits. The fat was probably attached to the intestines. The crude material (640 g.) was autoclaved to render it sterile, crushed and extracted with acetone. Removal of the solvent left the fat (580 g.) as a soft, white, solid with the following characteristics; saponification equivalent 288·5, iodine value 73·0, free fatty acid 0·3% (as oleic). The mixed acids resulting on hydrolysis had saponification equivalent 275·9 and iodine value 76·1.

Method of analysis

The mixed acids were divided into four less complex fractions by crystallization under various conditions, the insoluble acids being recrystallized in each case. Crystallization was effected from acetone at $-60°$ (twice), and from methanol at $-40°$ and at $-20°$. Five fractions, *A*, *B*, *C'*, *C''* and *D* in order of increasing solubility were thus obtained, *C'* and *C''* being combined to form fraction *C*. The amounts and iodine values of each fraction are given in Table 1.

Concentrations of 10 ml. of solvent/g. of acids are used throughout.

Table 1. *Low-temperature crystallization of python fat*

Fraction	*A*	*B*	*C*	*D*
Weight (g.)	54·06	66·61	26·14	35·26
Percentage of total	29·7	36·6	14·3	19·4
Iodine value	2·5	84·5	94·4	156·8

Each fraction was then methylated and fractionally distilled under reduced pressure, the iodine value and saponification equivalent of each fraction determined and certain fractions examined spectrographically, after

Table 2. *Fractionation of methyl esters of python fat*

Fraction	Wt. (g.)	Iodine value	Saponification equiv.	Fraction	Wt. (g.)	Iodine value	Saponification equiv.
A 1	2·90	0·2	267·4	*B*1	2·79	26·4	268·7
A 2	2·87	0·2	270·1	*B*2	2·85	70·4	290·2
A 3	3·13	0·2	270·8	*B*3	3·20	83·6	294·6
A 4	3·04	0·2	271·1	*B*4	3·24	84·8	295·2
A 5	3·86	0·1	270·7	*B*5	2·95	85·3	295·0
A 6	3·89	0·2	270·7	*B*6	3·04	85·3	294·9
A 7	3·56	0·2	271·0	*B*7	3·36	85·3	294·8
A 8	3·47	0·6	272·6	*B*8	3·42	85·6	295·4
A 9	3·60	5·0	285·6	*B*9	3·13	85·4	295·6
A 10	3·04	7·3	298·3	*B*10	3·16	85·2	295·1
A 11	3·70	5·9	299·2	*B*11	3·12	85·2	295·4
A 12	3·34	4·5	300·2	*B*12	3·34	85·3	295·7
A 13	4·96	3·4	299·5	*B*13	3·54	85·1	295·6
A 14*	2·96	9·7	324	*B*14	4·99	84·8	295·7
				*B*15	2·55	82·9	298·0
				*B*16†	1·25	67·5	300·5
*C*1	1·635	21·8	246·7	*D*1	2·65	68·0	255·0
*C*2	1·548	59·1	271·7	*D*2	2·73	95·2	271·5
*C*3	1·766	79·8	285·8	*D*3	3·11	128·9	285·8
*C*4	1·931	93·6	292·8	*D*4	3·70	151·9	294·6
*C*5	1·966	98·8	294·3	*D*5	2·80	154·8	294·6
*C*6	1·822	100·6	294·7	*D*6	2·97	154·7	294·4
*C*7	1·958	100·5	295·1	*D*7	3·23	153·9	295·2
*C*8	2·029	100·8	295·1	*D*8	3·35	152·7	294·7
*C*9	1·894	100·4	294·0	*D*9	3·18	152·8	295·6
*C*10	1·861	100·1	294·5	*D*10	3·17	177·8	306·4
*C*11	1·915	99·4	294·7	*D*11†	3·63	196·5	341
*C*12	2·276	98·6	301·5				
*C*13†	2·040	81·3	336				

* *A* 14 acids, excluding unsaponifiable, iodine value 9·2, saponification equiv. 298·7.
† Weight of unsaponifiable *B*16, 0·03 g.; *C*13, 0·09 g.; *D*11, 0·39 g.

Table 3. *Ultraviolet-absorption measurements* ($E^{1\%}_{1\,cm.}$)

		Unisomerized				Isomerized			
	λ (mμ.) ...	234	268	300	315	234*	268†	300†	315†
Fraction	Iodine value								
*B*8	—	—	—	—	—	Nil	Nil	Nil	Nil
C acids	94·4	—	—	—	—	120·0	4·0	Nil	Nil
*C*2	62·3	—	—	—	—	14·7	4·0	Nil	Nil
*C*8	105·8	9·9	Nil	Nil	Nil	152·1	Nil	Nil	Nil
*C*13	—	41·0	16·1	6·9	5·1	80·2	31·4	10·5	9·0
D acids	156·8	—	—	—	—	468·6	77·6	15·3	12·3
*D*2	100·8	6·5	Nil	—	—	82·0	2·9	—	—
*D*7	161·3	9·0	Nil	—	—	627·1	31·6	Nil	Nil
*D*11	216·3	89·7	52·7	18·5	13·8	350·0	319·8	113·3	99·2

* Isomerized at 180° for 60 min. † Isomerized at 170° for 15 min.

hydrolysis to the acids, both before and after alkali isomerization (Hilditch, Morton & Riley, 1945; Hilditch, Patel & Riley, 1951). Fractionation data are recorded in Table 2 and absorption measurements in Table 3.

Calculation of results

Fractions *A* and *B* were calculated by the standard methods described by Hilditch (1947, pp. 484–512); the methods used in calculating fractions *C* and *D* are briefly reported. The isomerization data for *C*8 (assumed to contain only C_{18} esters) indicates the absence of saturated and triethenoid esters; this fraction is accordingly calculated as a mixture of mono- and di-ethenoid esters on the basis of the observed iodine value. Fractions *C*6 to *C*11 are computed in the same way. C_{18} esters appearing in fractions before *C*6 are assumed to have the same composition as in *C*6 and C_{18} esters in fractions later than *C*11 as in this fraction (*C*11). The small absorption recorded at 234 mμ. for *C*2 is almost wholly due to the small quantity of C_{18} esters present in this fraction which is therefore taken as a mixture of palmitic, hexadecenoic and unsaturated C_{18} esters, as are fractions *C*3 and *C*4. The small amount of C_{16} esters in *C*5 is taken to be wholly palmitic. The composition of the C_{20} esters in fraction *C*12 is determined by the method of successive approximations, and the fraction is then partitioned between the C_{18} and C_{20} esters. The final fraction is similarly con-

sidered as a mixture of C_{18} and C_{20} esters along with unsaponifiable material.

Fraction *D* is calculated by methods similar to those described for fraction *C*. Assuming *D*7 to be wholly C_{18} esters and free of stearate (shown to be absent from the *C* fraction) its composition in terms of mono-, di- and triethenoid acids is calculated from the spectroscopic data. The results indicate the presence of conjugated and of traces of non-conjugable isomers of the octadecadienoic acid (cf. Barker & Hilditch, 1950). Fractions *D*5 to *D*9 are all considered to have the same composition as *D*7. *D*2 is a mixture of C_{16} and C_{18} esters; the composition of the latter is taken to be the same as in *D*5 and that of the former is obtained by the successive approximation procedure. These values for the C_{16} and C_{18} esters are then used to calculate *D*3 and *D*4 as mixtures of these esters. Fraction *D*1 is taken as myristic, tetradecenoic and mixed C_{16} esters with the same composition as in *D*2. Fractions *D*10 and *D*11 are calculated as already described for *C*12 and *C*13.

The separate results are finally gathered together in Table 4.

Identification of the acids present

The presence of the acids calculated to occur in this fat has been confirmed by the isolation of the acids, or of suitable derivatives, from appropriate ester fractions. Palmitic acid (m.p. and mixed m.p. 62–63°) and stearic acid (m.p. and mixed m.p. 69–71°) were isolated from fractions *A*4 and *A*10, respectively. Oxidation of fractions *D*2 and *B*8 with $KMnO_4$ in dilute alkaline solution gave respectively dihydroxypalmitic acid (m.p. 123·5–125·5°, undepressed when mixed with a similar sample from camel fat) and dihydroxystearic acid (m.p. and mixed m.p. 130–131°). Bromination of the acids from *D*7 afforded tetrabromostearic acid (m.p. and mixed m.p. 112–113·5°) and a little hexabromostearic acid (m.p. 175–178°, raised to 181–182° on admixture with an authentic specimen). The bromination was carried out quantitatively following the procedure described by Markley (1947), the results indicating the presence of 77% of linoleic acid. This is higher than the value calculated from the isomerization data (67%) but the use of polybromide numbers is known to give only approximate results. This high value indicates that the octadecadienoic acid present in python fat is almost entirely the linoleic acid present in seed fats.

RESULTS AND DISCUSSION

The final results obtained in this analysis are recorded in Table 5, along with comparable data for some other amphibians and reptiles.

Klenk (1933) has commented on the fact that the acid components of these fats are intermediate between those of fats obtained from marine sources and from higher land animals, and this view has been accepted by Hilditch (1947). The present work shows python fat to be clearly intermediate between the marine and the higher land animal fats. This is shown particularly by the following values (figures in parenthesis are average values for marine and

Table 4. *Component acids of python fat*

	A	*B*	*C*	*D*	Total	% (wt.)*	% (mol.)*
Myristic	0·19	0·07	0·62	0·40	1·28	1·3	1·6
Palmitic	16·77	1·87	0·77	0·24	19·65	19·7	21·2
Stearic	10·58	0·15	—	—	10·73	10·8	10·5
Arachidic	1·15	—	—	—	1·15	1·2	1·0
Tetradecenoic	—	—	0·15	0·30	0·45	0·5	0·5
Hexadecenoic	—	0·65	0·75	2·53	3·93	3·9	4·3
Hexadecadienoic	—	—	—	0·04	0·04	Trace	Trace
Octadecenoic	0·76	33·67	8·92	3·60	46·95	47·0	46·0
Octadecadienoic	—	—	1·79	8·85	10·64	10·7	10·5
Octadecatrienoic	—	—	—	0·75	0·75	0·8	0·7
As Eicosenoic†	0·17	0·15	1·30	2·43	4·05	4·1	3·7
Unsaponifiable	0·07	0·02	0·06	0·23	0·38	—	—

* Excluding unsaponifiable material.
† Includes all unsaturated acids higher than C_{18} (average unsaturation −4·1 H).

Table 5. *Component acids of amphibian and reptile fats*

(Values quoted are percentage by weight.)

	Python (*a*)	Frog (*b*)	Lizard (*c*)	Lizard (*d*)	Tortoise (*e*)
Myristic	1	4	4	4	1
Palmitic	20	11	18	29	14
Stearic	11	3	7	10	4
Arachidic	1	—	—	—	—
Hexadecenoic	4	15	10	12	9
Octadecenoic	59 (−2·4 H)	52 (approx. −2·5 H)	56 (−2·4 H)	40 (−2·7 H)	65 (−2·4 H)
Higher unsaturated acids	4 (−4·1 H)	15 (approx. −6 H)	5 (approx. −5 H)	5 (−5·5 H)	7 (approx. −4 H)

(*a*), Present work. (*b*), Klenk (1933). (*c*) and (*e*), Klenk, Ditt & Diebold (1935). (*d*), Hilditch & Paul (1937).

higher land-animal fats, respectively); palmitic acid, 20 % (12–15 %, 25–30 %); stearic acid, 11 % (very small, values rising to 30 %); and also in the mean unsaturation of the C_{18} unsaturated acids, $-2{\cdot}4$H. (Unsaturation more than $-3{\cdot}0$H, slightly greater than $-2{\cdot}0$H.) The content of hexadecenoic acid (4 %) and of unsaturated acids higher than C_{18} (4 %) are closer to the lower values typical of the higher land animals than to the higher values recorded for fish oils. The figures given in Table 5 show a general agreement between python fat and the other results given for frog, lizard and tortoise fats. It is of interest to note that fat from a frog, the only amphibian listed here, is more similar to that of the fishes than are the reptile fats.

Many analyses of animal fats, detailed in other respects, do not distinguish between unsaturated acids with the same carbon content; instead the average unsaturation is recorded. Following the use of low-temperature crystallization and of spectrographic methods as standard procedures in fat analysis, it has been possible to obtain greater insight into the nature of the unsaturated and, more particularly, the polyethenoid acids. Thus octadecadi- and octadecatri-enoic acids have been shown to occur in several animal fats. In python fat the unsaturated C_{18} acids include oleic (47·1 %), linoleic (10·7 %) and linolenic (0·8 %) acids, the nature of the polyethenoid acids being indicated by the isolation of tetrabromostearic acid (in high yield) and of hexabromostearic acid. Calculation of the spectrographic data actually indicates the presence of traces (1–2 % of the C_{18} diene acids) of isomeric octadecadienoic acids. These quantities are not considered large enough to be significant. Relatively larger quantities of these isomers have been reported in hippopotamus fat.

The lack of information concerning the detailed structure of polyethenoid acids higher than C_{18} still makes it difficult to distinguish individual acids among this group which are consequently grouped together as 'eicosenoic' acid. This group of acids has a mean unsaturation of $-4{\cdot}1$H and the spectrographic data (on fractions *C* 13 and *D* 11) indicate the presence of tetraethenoid acids; less unsaturated acids must also be present.

In calculating the results from spectrographic data obtained after alkali isomerization, empirical constants are used. These constants result from the study of pure acids obtained from seed fats by physical means (Hilditch *et al.* 1951) and thus apply to the wholly *cis*-acids. Under the standard conditions of isomerization, different constants would be obtained with acids having wholly or partly a *trans*-structure. The use of the published constants for animal fats therefore presupposes that the polyethenoid acids are identical with those from seed fats or at least that they behave identically on alkali isomerization. It is considered that the octadecadi- and octadecatri-enoic acids in python fat are essentially linoleic and linolenic acids so that the error introduced by using these constants is very small, if any. Further evidence that the octadecadienoic acid in another animal fat (crocodile) is ordinary linoleic acid has been obtained in a different way (Gunstone & Russell, 1952).

Our attention has been drawn to some recent comparative studies of bile salts which appear to lead to certain generalizations similar to those resulting from the study of component fatty acids. Haslewood & Wootton (1950) state that the nature of the bile salts appears to be a characteristic property of the species. Evolutionarily more primitive vertebrates contain C_{27} acids or alcohols, whilst more 'modern' vertebrates have C_{24} acids. In addition certain species, or groups of related species, elaborate unique bile salts which, according to Haslewood & Wootton (1950), is to be expected among more primitive species. The Boidae (boa and python) appear to belong to this last class in that their bile salts contain appreciable quantities of pythocholic acid (Haslewood & Wootton, 1951). More relevant to the discussion in this paper is the observation that the Crocodylidae (alligator and crocodile) which belong to the wider group of Amphibia and Reptilia under discussion contain a C_{27} acid, trihydroxycoprostanic acid, among their bile salts (Haslewood, 1952). The same acid has also been obtained from frog bile (Haslewood, 1952). The structure of this acid (II) clearly shows it to be intermediate in constitution between scymnol (I) (present in the bile of Elasmobranch fishes) and cholic acid (III) (present among the bile acids of higher land animals).

$$C_{19}H_{26}O_3.CHMe.CH_2.CH_2.\underset{\diagdown\,O\,\diagup}{CH.CMe}.CH_2OH, \quad (I)$$

$$C_{19}H_{26}O_3.CHMe.CH_2.CH_2.CH_2.CHMe.COOH, \quad (II)$$

$$C_{19}H_{26}O_3.CHMe.CH_2.CH_2.COOH. \quad (III)$$

SUMMARY

1. A quantitative study of the component acids of python fat is reported for the first time.

2. The results are similar to those previously reported for other amphibians and reptiles, thus providing additional evidence in support of the generalization that fats of this type are intermediate in composition between fish fats and land-animal fats.

3. Palmitic (19·7 %), stearic (10·8 %), oleic (47·1 %) and octadecadienoic (10·7 %) acids are major components, the octadecadienoic acid being almost entirely linoleic acid. Myristic, arachidic, tetradecenoic, hexadecenoic, octadecatrienoic and higher unsaturated acids are present as minor components.

We wish to thank Mr G. T. Iles of Belle Vue, Manchester, for supplying us with the sample of python fat used in this investigation, and for the information concerning the animal; also Prof. J. W. Cook, F.R.S., for drawing our attention to the work on bile salts; the University of Glasgow for a scholarship held by one of us (R.P.P.) and Imperial Chemical Industries Ltd., for a grant towards the purchase of apparatus.

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Enzymic Hydrolysis of Adenosine Phosphates by Cobra Venom

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Zeller (1948, 1950*a*) found that snake venoms liberate one molecule of inorganic orthophosphate (ortho-P) from adenosinetriphosphate (ATP), and concluded that the venoms contain an enzyme which hydrolyses ATP to adenosinediphosphate (ADP) and ortho-P. In an attempt to use the enzyme of cobra venom for the preparation of ADP from ATP Zeller's observation of the formation of one molecule of ortho-P from ATP was confirmed but no ADP was found. A further study of the reaction revealed that the enzyme, in fact, hydrolyses ATP to adenosine, inorganic pyrophosphate (pyro-P) and ortho-P, and that ADP is not an intermediate in this reaction.

EXPERIMENTAL

Materials and methods

Adenosine phosphates. The barium salt of ATP was prepared from rabbit muscle (see LePage, 1949) and converted into the sodium salt as described by Eggleston & Hems (1952). The concentration of ATP in the stock solution was about 6·5 mg./ml. The preparation contained some ADP, ortho-P and pyro-P; the quantities of these impurities are stated for each experiment. A sample of adenosinemonophosphate (AMP) (free acid) was supplied by Roche Products Ltd., through the courtesy of Dr F. Bergel and Dr A. L. Morrison. It showed no other phosphates or adenosine derivatives when tested by the chromatographic procedure described below. The concentration of the stock solution was 14 mg./ml. AMP, neutralized to pH 8·4. Barium-ADP was obtained from Schwarz Laboratories Inc., New York. It was doubly decomposed with Na_2SO_4 and was also found to be free from phosphates and other adenosine derivatives when tested chromatographically.

Cobra venom. A sample of dried cobra venom (*Naia naia*) was kindly supplied by the late Dr C. H. Kellaway, F.R.S. A stock solution of 5·4 mg./ml. was used in all experiments.

Antiserum. A sample of dried cobra 'antivenine' was also supplied by Dr Kellaway. This is a dried serum prepared by Lamb (1902, 1904) from the blood of rabbits which had received, about every 10 days for 4 or 5 months, subcutaneous injections of cobra venom. This serum forms a precipitate with cobra venom which is maximal when the ratio of venom to antiserum is 1 to 4.

Incubation and sampling. Stock solutions of cobra venom (0·2 ml.), adenosine phosphate (0·2 ml.) and 0·1 M-barbitone buffer (Michaelis, 1930), pH 8·4 (0·4 ml.), were mixed and incubated at 30°. When antiserum was included it was dissolved in the buffer solution. The pH chosen is near the optimum for the venom ATP-ase (Zeller, 1948, 1950*a*) and 5-nucleotidase (Gulland & Jackson, 1938). A sample (0·1 ml.) was taken immediately after mixing and transferred to 30% (w/v) aqueous trichloroacetic acid solution (0·05 ml.). Other samples were collected at intervals and treated in the same way. They were stored in the 'deep freeze' till they were analysed.

Chromatographic analysis. Separation of the adenosine derivatives and phosphates was carried out by chromatography. The method of Eggleston & Hems (1952) was used with certain modifications necessitated by the presence of

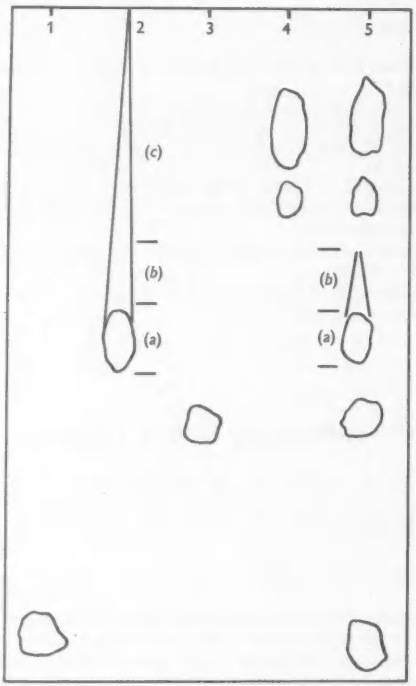

Fig. 1. A tracing of a chromatogram of phosphate compounds run in *n*-butanol-acetic acid-water for 60 hr. at 20°. (1) Ortho-P; (2) pyro-P; (3) AMP; (4) ADP (small spot) and ATP; (5) mixture. P content of the pyro-P 'spot', sectioned as shown, was as follows: (2*a*) 1·07; (2*b*) 0·54; (2*c*) 0·12 and (5*a*) 1·51; (5*b*) 0·20 μg. P.

Table 1. *Recovery of ATP and other phosphates from a mixture*

(For chromatographic procedure, see text. The quantities stated were contained in 5 μl. of water.)

Material	Amount of P (μg.) in material	
	Added	Recovered
Adenosinetriphosphate	1·81	1·88
Adenosinediphosphate	0·42	0·37
Adenosinemonophosphate	2·32	2·31
Pyro-P	1·73	1·71
Ortho-P	0·21	0·22

pyro-P, which was found to have about the same R_P value as ADP in the solvents of Eggleston & Hems. When these were replaced by a *n*-butanol-acetic acid-water mixture (40:10:50, v/v), a mixture of ATP, ADP, AMP, adenosine,

Table 2. *Decomposition of ATP and ADP by cobra venom*

(Incubation at 30° in 0·05 M-barbitone buffer (pH 8·4). Venom, 0·14% (w/v). Vol. of mixture, 0·8 ml. 'Observed change' refers to the increase above the zero value. 'Calculated change' is based on the assumption that ATP yields equimolar amounts of adenosine, ortho-P and pyro-P, and that ADP yields one equivalent of adenosine and two of ortho-P.)

Material	Amount of material (μmoles) at									
	0 hr.	0·5 hr.			1·5 hr.			4 hr.		
	Found	Found	Observed change	Calculated change	Found	Observed change	Calculated change	Found	Observed change	Calculated change
Adenosinetriphosphate	1·44	1·15	−0·29	—	0·19	−1·25	—	0	−1·44	—
Adenosinediphosphate	0·60	0·44	−0·16	—	0·24	−0·35	—	0	−0·60	—
Adenosinemonophosphate	0	0	0	—	0	0	—	0	0	—
Pyro-P	0·04	0·39	0·35	0·29	1·10	1·06	1·25	1·35	1·31	1·44
Ortho-P	0·74	1·27	0·53	0·61	2·56	1·82	1·97	3·48	2·74	2·64
Adenosine	0	0·63	0·63	0·45	1·77	1·77	1·61	2·12	2·12	2·04

ortho-P and pyro-P separated when run in the downward direction for 50 hr. None of the substances decomposed under these conditions. After about 30 hr. the adenosine spots approached the bottom end of the paper where they were located by ultraviolet light. To obtain the adenosine values the section of the paper containing the substance was cut off whilst the strip was still wet with solvent. The 'run' was continued, and after 50–60 hr. the ortho-P reached the bottom of the shortened paper. The excised section was air-dried, the adenosine was eluted with 3 ml. 0·1 M-phosphate buffer, pH 7·0, at 20° for about 30 min. and the absorption of the eluate at 260 mμ. was measured spectrophotometrically. When P analyses were not required the chromatogram was run for 28 hr. and the adenosine compounds removed and estimated as described above for adenosine. It was sufficiently accurate to estimate ATP and ADP together as ATP, since the P content affects the molar extinction coefficients of such compounds only slightly (see Kalckar, 1947), and the proportion of ADP was small.

Table 3. *Effect of antiserum on the decomposition of ADP by cobra venom*

(Incubation at 30° in 0·02 M-barbitone buffer (pH 8·4). Venom, 0·10% (w/v). Antiserum, 2·0 mg. Vol. of mixture, 0·5 ml.)

Material	Amount of material (μmoles) at 0 hr.	4 hr.	21 hr.
(*a*) Without antiserum			
Adenosinediphosphate	2·76	0·77	0·15
Adenosinemonophosphate	0	0	0
Pyro-P	0	0	0
Ortho-P	0	4·24	5·52
(*b*) With antiserum			
Adenosinediphosphate	2·75	1·47	0·18
Adenosinemonophosphate	0	1·11	0·42
Pyro-P	0	0	0
Ortho-P	0	1·47	4·75

A tracing of a 60 hr. chromatogram of a mixture of adenosine derivatives and phosphates is shown in Fig. 1. Table 1 shows the satisfactory recovery of the added substances. It is noteworthy that pyro-P tails badly when run alone but tailing is so much decreased when a mixture of phosphates is present that separation offers no difficulty.

Nomenclature. According to a suggestion of Dr W. Klyne, Dr R. S. Cahn and Dr R. K. Callow the three P-atoms of ATP are distinguished as follows:

adenosine—P$^{\alpha}$—O—P$^{\beta}$—O—P$^{\gamma}$.

RESULTS

Hydrolysis of adenosine phosphates by cobra venom. The time curves of the effect of cobra venom on ATP and ADP are shown in Tables 2 and 3. The column '0 hr.' gives the composition of the starting material. All ATP and such ADP as was present were completely dephosphorylated after 4 hr. in the experiment recorded in Table 2. As the concentrations of ADP and ATP fell those of adenosine, pyro-P and ortho-P rose. No AMP appeared. The data support the assumption that ATP is split into one molecule each of adenosine, pyro-P and ortho-P, and ADP into adenosine and 2 molecules of ortho-P. The pyro-P found was slightly below, and the ortho-P slightly above, the expected values, which can be accounted for by slow hydrolysis of pyro-P (see below). The hydrolysis of ADP (Table 3) is slower than that of ATP. After 21 hr. 95% of the added ADP had disappeared under the test conditions and the amount of ADP removed and ortho-P formed were approximately in the expected proportion of 1:2. No pyro-P or AMP appeared.

Table 5. *Decomposition of inorganic pyrophosphate by cobra venom*

(For conditions, see Table 2.)

Material	Amount of material (μmoles) at 0 day Found	6 days Found	6 days Observed change
Pyro-P	2·8	1·2	−1·6
Ortho-P	0	3·0	3·0

AMP was rapidly hydrolysed to adenosine and ortho-P (Table 4), the initial rate of disappearance of AMP being about 5 times higher than that of

Table 4. *Effect of antiserum on the decomposition of AMP by cobra venom*

(Incubation at 30° in 0·05 M-barbitone buffer (pH 8·4). Venom, 0·14% (w/v). Antiserum, 4·4 mg. Vol. of mixture, 0·8 ml.)

Material	Amount of material (μmoles) at 0 hr.	0·5 hr.	1·5 hr.	4 hr.	7 hr.	24 hr.
(*a*) Without antiserum						
Adenosinemonophosphate	7·90	2·10	0·05	0	—	—
Ortho-P	0	6·15	8·18	8·24	—	—
Adenosine	0	5·77	6·93	7·55	—	—
(*b*) With antiserum						
Adenosinemonophosphate	8·32	—	7·42	6·67	5·35	2·01
Ortho-P	0	—	1·04	1·38	2·91	6·17
Adenosine	0	—	0·44	1·09	2·02	5·78
						40·2

Table 6. *Effect of antiserum on the decomposition of ATP by cobra venom*

(For conditions see Table 4.)

Material	Amount of material (μmoles) at 0 hr.	0·5 hr.	1·5 hr.	4 hr.	21 hr.
	(*a*) Without antiserum				
Adenosinetriphosphate	2·06	0·89	0·18	0	—
Adenosinemonophosphate	0·18	0	0	0	—
Adenosine	0	1·28	1·69	2·32	—
	(*b*) With antiserum				
Adenosinetriphosphate	2·24	1·48	0·86	0·03	0
Adenosinemonophosphate	0·21	0·81	1·62	1·85	0
Adenosine	0	0	0	0·50	2·38

Table 7. *Location of* ^{32}P *before and after decomposition of* $[\beta\gamma\text{-}^{32}P_2]$ *ATP and* $[\beta\text{-}^{32}P]$ *ADP by cobra venom*

(For conditions see Table 2. 'Observed change' refers to the increase above the zero value. 'Calculated change' is based on the assumption that ^{32}P in $[\beta\gamma\text{-}^{32}P_2]$ ATP appears as pyro-P and that ^{32}P in $[\beta\text{-}^{32}P]$ ADP appears as ortho-P.)

Material	Amount of ^{32}P (counts/min./0·01 ml. of incubated mixture) in material at 0 hr. Found	4 hr. Found	Observed change	Calculated change
Adenosinetriphosphate	212	61	−151	—
Adenosinediphosphate	41	11	−30	—
Pyro-P	20	175	155	151
Ortho-P	23	46	23	30

ATP. This confirms the presence of a highly active 5-nucleotidase in cobra venom (Gulland & Jackson, 1938; Zeller, 1950*b*).

Hydrolysis of inorganic pyrophosphate. The yields of pyro-P and ortho-P from ATP (see Table 2) suggest that the venom contains a weak pyrophosphatase. This is borne out by the experiment recorded in Table 5. Recrystallized $Na_4P_2O_7 . 10H_2O$ (0·2 ml., 4·0 mg./ml.) was incubated under the same conditions as ATP. After 6 days a little above 50 % of the added pyro-P was hydrolysed to ortho-P (Table 5). There was no hydrolysis in the absence of cobra venom under the same conditions. An inorganic pyrophosphatase is thus present in cobra venom but its activity is slight compared with the ATP-ase and 5-nucleotidase.

Action of venom on adenosine. After incubation for several weeks with cobra venom in the presence of chloroform, adenosine remained unchanged and no ribose, adenine or inosine was formed.

Effect of antiserum. Addition of antiserum (4·4 mg. in 0·8 ml. mixture) reduced the rate of disappearance of ATP by about one-third (Table 6). The formation of adenosine was negligible during the first 90 min.; instead AMP accumulated. Antiserum alone did not attack ATP. Dried serum from the normal rabbit had no inhibitory effects on the action of cobra venom.

The observations suggest that two enzymes are concerned with the hydrolysis of ATP, an ATP-ase, which converts ATP into AMP and pyro-P and a 5-nucleotidase, which hydrolyses AMP to adenosine and ortho-P. The antiserum inhibits the latter enzyme more powerfully than the former (see Table 4 and 6); it also inhibits the decomposition of ADP (Table 3).

Decomposition of isotopic ATP. ATP, prepared according to Hems & Bartley (1953) and in which the β- and γ-P atoms were replaced by ^{32}P, was incubated with cobra venom in the absence and presence of antiserum. The AMP which accumulated during the first 4 hr. in the presence of antiserum showed no radioactivity. In the absence of antiserum practically all of the radioactivity was located in the pyro-P (Table 7). This confirms the conclusions reached in the preceding paragraph.

DISCUSSION

The observations on the effect of cobra venom on adenosine derivatives are all accounted for by the assumption that the following reactions occur in the presence of the venom.

(1) ATP → AMP + pyrophosphate (rapid).

(2) AMP → adenosine + orthophosphate (very rapid).

(3) pyrophosphate → 2 orthophosphate (very slow, rate about 1 % of that of (1)).

(4) ADP → adenosine + 2 orthophosphate (slow, rate about 10 % of that of (1)).

As (2) is much more rapid than (1) AMP does not accumulate normally, but when (2) is selectively inhibited by antiserum AMP accumulates.

The enzyme-catalysing reaction (1) hydrolyses the $\alpha\beta$-pyrophosphate bond of ATP and may therefore be described as $\alpha\beta$-ATP-ase, in contrast to the common ATP-ases of animal tissues and other materials, which are $\beta\gamma$-ATP-ases. The enzyme of reaction (2) is the 5-nucleotidase of Gulland & Jackson, which removes phosphate from AMP and inosine 5-phosphate but not from 3-phosphate nucleotides. Reaction (4) is presumably the result of a combined action of an ADP-ase and 5-nucleotidase.

The fission of ATP into equimolar quantities of adenosine, ortho-P and pyro-P has already been recorded, though without experimental details, by Hitchings & Fuller (1939). Zeller (1950*b*) believed that this occurred only 'under certain conditions, e.g. low substrate concentration'. The venom investigated in the present work did not, as Zeller (1951) assumed, transform ATP into ADP or hydrolyse the latter to adenosine and pyrophosphate.

The observation of Bhattacharya & Ghosh (1951) that one equivalent of acid is formed when ADP is decomposed by cobra venom is explained by reaction (1). The known data for the ionization constants of the reactants (Alberty, Smith & Bock, 1951) indicate that the hydrolysis of one pyrophosphate bond yields about one equivalent of acid at pH 7·4.

As Zeller (1951) has pointed out, it is very probable that the $\alpha\beta$-ATP-ase and 5-nucleotidase of cobra venom are, like the nucleotide pyrophosphatase (Chain, 1938, 1939), components of the toxic principle of the venom. Cobra venom is known to depress the blood pressure, and Kellaway & Trethewie (1940) have demonstrated the release by cobra venom *in vivo* of 'adenyl compounds' (i.e. substances depressing the blood pressure in the atropinized guinea-pig heart preparation). Two of the products of the decomposition of ATP, namely adenosine and pyrophosphate, have such depressor effects (Green & Stoner, 1950; Bielschowsky & Green, 1944). Thus by two enzyme-catalysed reactions the key metabolite of animal tissues, ATP, is converted into two toxic substances. Moreover, unlike other breakdown products of ATP, adenosine and pyrophosphate cannot be readily resynthesized to ATP by the enzyme systems responsible for oxidative phosphorylation. It remains to be investigated whether the same or different enzymes cause the destruction of nucleotide coenzymes by snake venoms (Chain, 1939; Fleckenstein & Gayer, 1950).

SUMMARY

1. The effect of cobra venom on adenosine phosphates has been studied. The products formed were separated and estimated chromatographically.

2. In the presence of cobra venom, adenosinetriphosphate is almost quantitatively converted into adenosine, orthophosphate and pyrophosphate; adenosinemonophosphate is converted into adenosine and orthophosphate.

3. Antiserum inhibits the hydrolysis of adenosinetriphosphate. In the presence of antiserum, no adenosine is formed in the earlier stages of incubation; instead adenosinemonophosphate appears.

4. Adenosinetriphosphate labelled with ^{32}P in the β and γ positions (for nomenclature see p. 627) yields radioactive pyrophosphate and inactive orthophosphate when incubated with cobra venom.

5. The findings indicate that the venom contains an adenosinetriphosphatase which hydrolyses the $\alpha\beta$-pyrophosphate bond, differing from the common tissue adenosinetriphosphatases which hydrolyse the $\beta\gamma$-bond. This enzyme together with the 5-nucleotidase described by Gulland & Jackson (1938) account for the hydrolysis of adenosinetriphosphate.

6. Adenosinediphosphate is slowly hydrolysed by the venom to adenosine and two molecules of orthophosphate. No pyrophosphate is formed.

7. The role of the above enzymes in the toxic action of cobra venom is discussed.

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Investigations of D-Homosteroids as Potential Carcinogens

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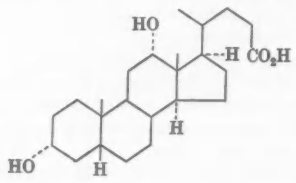
The high carcinogenic potency of 20-methylcholanthrene and its close structural relationship to the steroids led to the hypothesis that carcinogenic hydrocarbons might arise from endogenous steroids under abnormal metabolic conditions.

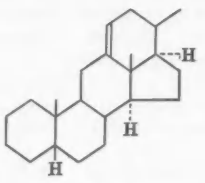
In its original form this hypothesis envisaged the conversion of cholesterol or deoxycholic acid (I) via dehydronorcholene (II) into 20-methylcholanthrene (III), a process attainable by pyrolytic methods in the laboratory (Wieland & Dane, 1933; Fieser & Newman, 1935; Rossner, 1937), under physiological conditions. It was supported by the reported carcinogenic activity of benzene extracts of *Escherichia coli* cultures grown in presence of dehydronorcholene (Druckrey, Richter & Vierthaler, 1941); this activity cannot, however, be due to some transformation product, since dehydronorcholene, which is non-carcinogenic, is unaltered by *Esch. coli* (Butenandt & Dannenberg, 1950).

An alternative form of the hypothesis is suggested by the fact that the structural analogy between a cholanic acid (I) and 20-methylcholanthrene (III) is paralleled by the even closer analogy between 17*a*-methyl-*D*-homoandrostane (V) and 3-methylchrysene (VI).

The normal activity of the adrenal cortex leads to 17α-hydroxy-20-ketosteroids (as IV: R = H or OH); these are known to undergo facile conversion to derivatives of 17*a*-methyl-*D*-homoandrostane (V) by adsorption at a moist, neutral aluminium oxide surface at 20° (Stavely, 1940; Prins & Reichstein, 1940; Shoppee & Prins, 1943). Whilst there is no evidence that the dehydrogenation of derivatives of 17*a*-methyl-*D*-homoandrostane (V) to derivatives of 3-methylchrysene (III), accomplished *in vitro* (Ruzicka & Meldahl, 1940), occurs *in vivo* (cf. the



I



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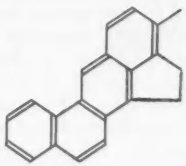

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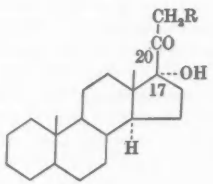

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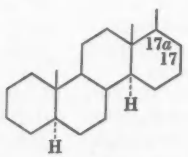
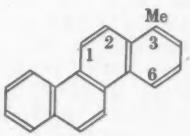


V



VI

dehydrogenation (II) → (III)), analogies may be quoted, e.g. the biological dehydrogenation of *cyclo*hexanecarboxylic acid to benzoic acid (Bernhard & Caflisch-Weill, 1945; Dickens, 1947; Beer, Dickens & Pearson, 1949) and of (+)-oestrone to (+)-equilenin in the pregnant mare (Girard, Sandulesco, Fridenson, Gaudefroy & Rutgers, 1932; Girard, Sandulesco, Fridenson & Rutgers, 1932). Further, although chrysene is non-carcinogenic, some of its homologues are active; thus 1:2-dimethylchrysene is carcinogenic (Hewett, 1940).

There is a considerable body of evidence that the adrenal cortex may be implicated in tumorigenesis (cf. Woolley & Little, 1945), and it has been observed that adrenocortical hormone production and metabolism are very frequently disturbed in neoplastic disease (Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948). If the facile change (IV → V) (cf.

Hirschmann & Hirschmann, 1947) can occur *in vivo* by adsorption at a lipoprotein surface, then in hyperfunction of the adrenal cortex *D*-homosteroids might arise in such quantity as to compel the organism to resort to abnormal metabolic paths; one path could lead to derivatives of 3-methylchrysene (VI). It seemed therefore of interest to prepare some *D*-homosteroids and some derivatives of 3-methylchrysene, and to test them for carcinogenic activity. Owing to external circumstances, the work had to be discontinued, and the present account deals with the simplest representatives, 17*a*-methyl-*D*-homoandrostane and 3-methylchrysene.

17*a*-Methyl-*D*-homoandrostane is not carcinogenic in stock mice when repeatedly applied to the skin in 0·5 % solution in benzene. 3-Methylchrysene appears to be a weak carcinogen, for although it produced no tumours in mice by painting, by the graft technique of Dr Horning (Royal Cancer Hospital) it caused proliferation of the prostatic epithelium with active mitosis in the glandular epithelium and infiltration of the stroma, processes which precede tumour formation.

EXPERIMENTAL

Melting points were determined on a Kofler block (limit of error $\pm 2°$). Solvents for chromatographic operations were rigorously purified and dried and, unless stated otherwise, aluminium oxide (Spence type H, activity II) was used. For drying of ethereal extracts, brief treatment with anhydrous sodium sulphate was used.

17a-*Methyl*-D-*homoandrostane*. This was obtained by Wolff-Kishner reduction of 17*a*-methyl-*D*-homoandrostan-3-one, m.p. 182° (Ruzicka & Meldahl, 1940; Shoppee & Prins, 1943). After purification by chromatography, repeated crystallization from $CHCl_3$: methanol, and sublimation at 130–140°/0·01 mm., it had m.p. 108–109°.

3-*Methylchrysene*. This was obtained by combination of procedures due to Bachmann & Struve (1940) and Cook & Schoental (1945). α-Tetralone with amalgamated zinc and methyl γ-bromocrotonate (Ziegler, Schumann, Winkelmann, Späth & Schaaf, 1942) gave methyl γ-1-(3:4-dihydronaphthyl)crotonate, isomerized by palladium black (prepared according to Heilbron, Sexton & Spring, 1929) at 280° for 3 hr. in an atmosphere of CO_2, and hydrolysed by 4% ethanolic KOH to γ-1-naphthylbutyric acid, m.p. 97–98°. This was cyclized directly with anhydrous HF (cf. Fieser & Hershberg, 1939; Fieser & Johnson, 1940) or as the chloride with $SnCl_4$ (cf. Wilds, 1942) to yield 1-keto-1:2:3:4-tetrahydrophenanthrene, m.p. 93°. Repetition of this series of reactions with the last named ketone gave seriatim methyl γ-1-(3:4-dihydrophenanthryl)crotonate, γ-1-phenanthrylbutyric acid, m.p. 155–157°, and 3-keto-3:4:5:6-tetrahydrochrysene, m.p. 229–230°, undepressed by a specimen, m.p. 228–229°, prepared by Bachmann & Struve (1940).

The tetracyclic ketone by treatment with methyl magnesium iodide, and simultaneous dehydration and dehydrogenation of the resulting carbinol with palladized charcoal (prepared according to Newman & Zahm, 1943) for 1 hr. at 300° in an atmosphere of CO_2 gave 3-methylchrysene; this was crystallized from benzene, further purified by filtration of a benzene solution through a column of aluminium oxide, and recrystallization from benzene, m.p. 253–254°, undepressed by admixture with a specimen, m.p. 250°, prepared by Bachmann & Struve (1940).

SUMMARY

The possible conversion of 17α-hydroxy-20-ketosteroids derived from the adrenal cortex into *D*-homosteroids and so into derivatives of 3-methylchrysene is discussed. *D*-Homoandrostane is non-carcinogenic, but 3-methylchrysene, prepared by a simple route from α-tetralone, is a weak carcinogen.

We wish to thank Prof. J. W. Cook, F.R.S., and Dr Schoental, for carrying out a cyclization with anhydrous HF, and Dr Horning and Mr Marsh of the Chester Beatty Research Institute for carrying out the biological tests. We acknowledge the gift of specimens of 3-keto-3:4:5:6-tetrahydrochrysene and of 3-methylchrysene by the late Prof. W. E. Bachmann. One of us (N.G.) wishes also to acknowledge a grant from the Medical Research Council which enabled her to participate in this work.

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The Fluorescence of Teeth under Ultraviolet Irradiation

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(*Received* 13 *January* 1953)

The observation that teeth fluoresce when irradiated with ultraviolet light was reported by Stubel (1911). He studied a whole series of tissues and found that the teeth exhibited an intense blue fluorescence second only in brilliance to the lens of the eye. Some years later, Benedict (1928) noted that dentine fluoresced much more strongly than enamel and with a seemingly bluer light. He observed that the white spot indicative of 'beginning' dental caries did not fluoresce. He produced a similar effect by scratching through a paraffin-coated tooth and placing in dilute acetic acid overnight. Benedict also noted that ashed enamel did not fluoresce, nor did dentine which had been boiled in 50 % sodium hydroxide, whilst on decalcifying dentine with dilute nitric acid the remaining organic matrix retained its fluorescence to an appreciable extent. Tiede & Chomse (1934) were of the opinion that it was the organic protein compounds of the teeth which were responsible for the phenomenon. They said that teeth extracted with alkaline glycerol retained enough organic matter to fluoresce, but that the property was lost after treatment with hydrogen peroxide and ammonia. Glasser & Fonda (1938), on the other hand, believed that the fluorescence was linked with the mineral phase. They used ox teeth, presumably the enamel only, since they stated that their material contained 1 % protein. According to these workers the fluorescence was destroyed by burning out the organic content at 600° but was increased by heating at 400°. They found that after 'solution of the teeth in acid and precipitation with alkali' the fluorescence was restored by heating at 400°. Bodecker (1939) put forward the suggestion that dental lymph was responsible for the fluorescence, although vital and non-vital teeth both fluoresce. Hadjioloff & Russewa (1943) observed that enamel, dentine and cementum appeared blue under ultraviolet light and that enamel and dentine sometimes appeared yellow. They reported that by decalcifying with a half-saturated solution of sodium chloride in dilute hydrochloric acid the teeth lost their ability to fluoresce.

The phenomenon of tooth fluorescence poses two main questions. First, what substance or substances are responsible and, secondly, why does the carious lesion cease to fluoresce? The answer to these two questions may be of fundamental importance in the aetiology of dental caries. If, for example, the active material should prove to be organic in nature, it would mean that disintegration of organic matter occurs at a very early stage in the carious process. The object of the present investigation was to observe the fluorescence in various tooth specimens and to explore techniques for extracting the active substances from the teeth, with a view to finding some information concerning their chemical nature.

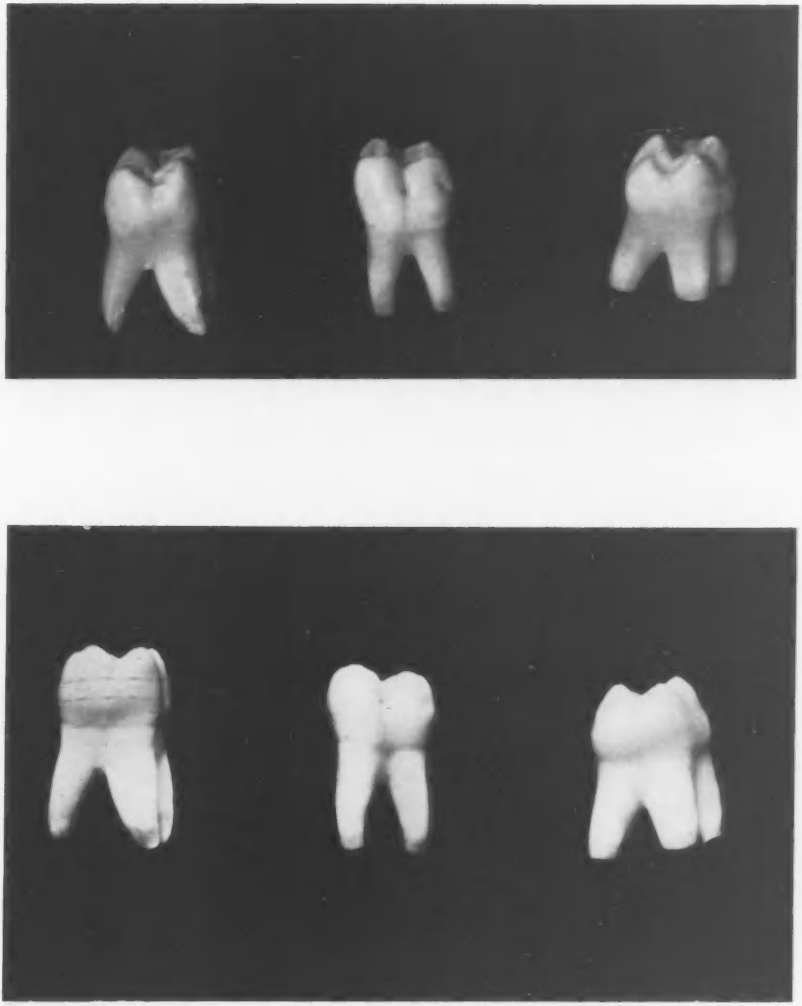
EXPERIMENTAL

Observations on the fluorescence of teeth

A 'dark bulb' mercury-vapour lamp was used routinely as a source of ultraviolet radiation. The appearance of the fluorescence was compared with that produced by a 'Hanovia' fluorescence lamp, Model 11, with Wood's glass filter which transmits in the region of 366 mμ. No differences were detected. Prior to their examination, teeth were freed from adhering tissues, calculus and stains by careful use of brush and pumice followed by a thorough washing in water. Observations were made on whole teeth and on sections.

Sound teeth

(i) *Enamel.* Enamel normally fluoresced bluish white but occasionally tinged with yellow. Pl. 2 shows a group of three sound teeth photographed under artificial white light and under ultraviolet light; the differences in colour intensity are lost in the black and white print; but an impression can be gained of the lustre of the fluorescent light.



A group of three teeth photographed in artificial white light (above) and in ultraviolet light (below).

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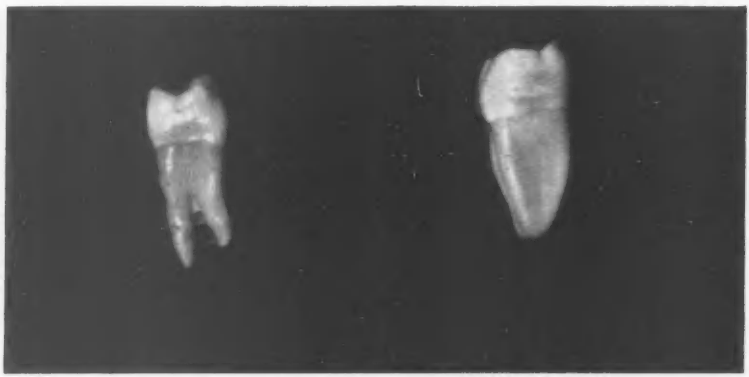
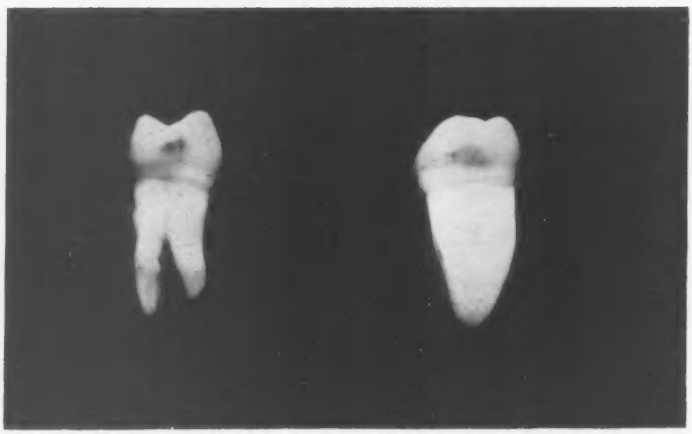
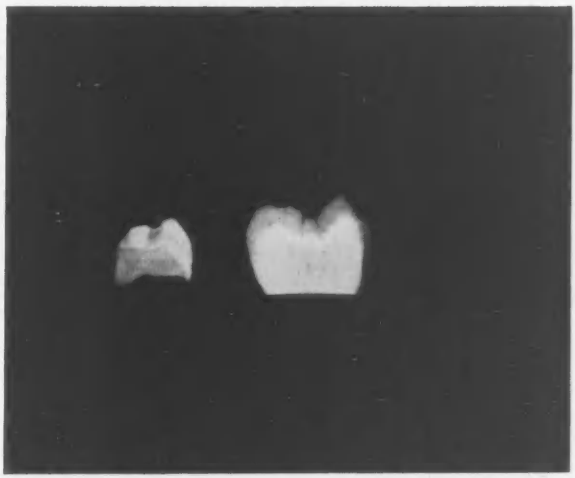




Early carious lesions showing as whitish patches when photographed in artificial white light (above), and as black non-fluorescent areas when photographed in ultraviolet light (below).



Photograph of two tooth sections in ultraviolet light. That on the left has been refluxed with ethylene glycol containing 3% potassium hydroxide, the intensity of the dentine fluorescence is markedly reduced compared with the normal section on the right.

(ii) *Dentine.* Dentine fluoresced much more strongly than did the enamel with an intense blue-violet light. The colour was most intense in the apical regions, particularly in some immature teeth with open apices.

(iii) *Cementum.* The appearance of the fluorescence was similar to that of dentine but the colour was less intense.

Carious teeth

(i) *Carious enamel.* This did not fluoresce, the carious areas appearing black. In the early lesions the whitish patches had lost their fluorescence (Pl. 3). In section, the loss of fluorescence extended with the lesion.

(ii) *Carious dentine.* The blue-violet colour was absent and the surfaces of the lesion appeared black or dark brown. When the carious dentine was carefully ground away an orange-brown fluorescence was observed, which at the base of the lesion, after removal of practically all the carious material, became pinkish orange in colour.

(iii) *Calculus.* Patches of calculus on the teeth could be distinguished readily as they fluoresced with a reddish orange colour.

The effect on tooth fluorescence of etching with dilute acid

We repeated Benedict's (1928) experiment of coating a tooth completely with paraffin wax, scratching through the coat to the enamel and immersing in 2N-acetic acid overnight. The tooth was then removed, washed and the wax scraped away. The scratch appeared as a black line when the tooth was viewed in ultraviolet light. The same result was obtained with 2N-HCl. If a drop of the dilute acid was placed carefully on the smooth surface of enamel, or if the tip of the enamel-coated crown was dipped into the acid and left overnight in a closed vessel no effect was noticed on the fluorescence. We are of the opinion that the dark appearance of the waxed etched tooth is a combination of optical factors and the forcing into the scratch of wax during the scraping process. A similar effect can be produced by scratching a waxed tooth with a fine glass cutter and then scraping off the wax.

The fluorescence of dentine

Dentine contains much more organic material and fluoresced much more strongly than the enamel. It is probable therefore that the fluorescing substances in dentine are associated with the organic fraction.

Ethylene glycol extraction of dentine. A longitudinal section through the crown of a tooth was placed in ethylene glycol containing 3% (w/v) KOH, a process used for extracting organic matter from dentine (Crowell, Hodge & Line, 1934), and refluxed for 90 min. The section was then removed, washed thoroughly with water until the washings were no longer alkaline to phenolphthalein and dried. On examining under ultraviolet light the fluorescence of the enamel was little changed in appearance but that of the dentine was very much weaker and less blue in colour (Pl. 4). The alkaline ethylene glycol fluoresced with a strong blue colour. It appeared therefore that this treatment, in removing the organic fraction of the dentine, had also extracted some fluorescing substances. It was decided therefore to extract a larger amount of dentine.

The crowns were removed from a number of teeth and the roots stripped of adventitious material. Any caries, calculus and stains were removed with a bur, brush and pumice followed by washing in water. The contents of the root canal were removed with a dental bur. About 100 g. of the roots so prepared were split into fragments and ground in a percussion mortar until they passed through a 30-mesh sieve. Analysis of samples of this powdered dentine (plus a little cementum) showed that it contained 3·5% N (Kjeldahl). The dentine (100 g.) was then refluxed for 3 hr. with 250 ml. of ethylene glycol containing 3% (w/v) KOH. It was necessary to stir the mixture mechanically during the heating to avoid bumping and uneven heating. This process was in effect hydrolysis by dilute alkali at a high temperature (180–200°). After refluxing, the mixture was dark amber in colour but the liquid phase exhibited strong fluorescence in the meniscus or when diluted with water. The supernatant liquid was decanted and the residue washed about ten times with 50 ml. portions of boiling water until the washings were no longer pigmented and fluoresced only weakly. Samples of the extracted dentine now contained less than 0·05% N. The ethylene glycol extract plus the washings were centrifuged and filtered through an asbestos pad to remove suspended particles. The resultant solution was clear, of a deep-amber colour and fluoresced strongly at the surface. A drop of the solution placed on a filter paper produced an intensely blue fluorescing spot. Nitrogen determinations on the solution showed that it contained approximately 66% of the N originally present in the dentine.

This leaves more than 30% of the N unaccounted for. A strong ammoniacal and 'amine' smell was noticed during the digestion. In order to investigate these volatile compounds and to try and obtain a nitrogen balance sheet, a small quantity of dentine (1·7 g.) was boiled gently in alkaline ethylene glycol for 90 min.; the volatile compounds evolved were led through an excess of dilute H_2SO_4 to trap any basic material. The resulting solution was evaporated and was found to contain 15% of the N originally present in the dentine. The glycol extract contained 73% and the washed residues 2·6% of the total N. This heating was rather more gentle than the previous vigorous refluxing and the dentine was less thoroughly extracted; even so, only 90% of total N was recovered. We cannot yet account for the missing 10% except to suppose that it is evolved as volatile compounds not absorbed by dilute H_2SO_4.

Treatment of ethylene glycol extract. The diluted ethylene glycol extract was acidified with HCl to pH 1, shaken at room temperature with 10 g. of activated charcoal (British Drug Houses Ltd.) and filtered. This treatment removed most of the pigment and the fluorescence of the solution was markedly diminished. Two further treatments with charcoal rendered the filtrate colourless and almost non-fluorescent. The fluorescing materials and the pigments in the extract had therefore been adsorbed on the charcoal. The charcoal was washed thoroughly with successive quantities of cold water until the washings were non-fluorescent. Very little fluorescent material was removed by this treatment.

Elution from charcoal. Repeated extraction with 92% (v/v) ethanol produced a pale-yellow solution fluorescing blue, but the active materials were only removed slowly. The most satisfactory eluant for recovering the adsorbed

substances was a solution containing 5 vol. ethanol, 4 vol. water and 1 vol. of ammonia (sp.gr. 0·88). This produced a deep-amber-coloured eluate which fluoresced strongly. Unfortunately, some pigmented impurities already in the charcoal were also eluted by this treatment, but most of them could be removed later as they were relatively insoluble in absolute ethanol.

Treatment of eluate. The combined ammoniacal eluates were taken to dryness under reduced pressure at 50–60°, yielding a yellow amorphous solid (2·5 g.) containing 8·2% N (0·55% N, referred to 100 g. of original dentine). Thus about 15% of the total dentine nitrogen is contained in the eluate. The amorphous solid was dissolved in 50 ml. absolute ethanol and termed solution *A*. (A small amount of material, mainly impurities from the charcoal, did not dissolve.) The solid was also soluble in water and in methanol, partially soluble in acetone, but insoluble in ether, chloroform or light petroleum.

Paper chromatography. Chromatograms using both butanol/acetic acid and propanol/ammonia systems showed the presence of ninhydrin-positive material (i.e. amino-acids or peptides) but resolution was poor. The fluorescing substances tended to 'streak' the whole length of the chromatogram with areas of concentration not necessarily associated with ninhydrin-positive material.

A portion of solution *A* was again taken to dryness and the solids hydrolysed by refluxing with 6N-HCl for 8 hr. The hydrolysate (strongly fluorescent) was taken to dryness and extracted with successive small amounts of absolute ethanol, until finally only a small non-fluorescing ethanol-insoluble residue remained. The combined extracts (solution *B*) were centrifuged to remove small particles of suspended matter. Paper chromatograms of this solution showed the presence of several amino-acids normally present in dentine which were identified by comparison with chromatograms of known amino-acids running adjacently. The fluorescing materials were still not resolved although at high concentration there was a tendency to form crescents at a fairly constant R_F value. There was no correlation between the position of the amino-acids and the fluorescence.

Adsorption chromatography. Solution *B*, diluted with ethanol, was passed through a column of activated alumina (Peter Spence and Co. Ltd.). On developing with absolute ethanol a dark-brown band stayed at the top of the column but much fluorescent material passed through. Development was continued until the eluate was almost non-fluorescent. The eluate, when concentrated, was bright yellow and fluoresced strongly with a bluish colour. This eluate was taken to dryness and yielded a yellowish gum soluble in water, methanol and ethanol but insoluble in ether, chloroform and light petroleum. The gum was redissolved in methanol and to this solution was added an ethereal solution of diazomethane (prepared from nitrosomethylurea) and left to stand overnight. On evaporating to dryness the residue was almost completely soluble in $CHCl_3$ and still fluoresced strongly. The yellow $CHCl_3$ solution was now passed through an alumina column, whereby four distinct fluorescent zones developed and each was collected separately as it was washed off the column. When no further fluorescent material could be obtained by washing with $CHCl_3$, development was continued with ethanol. By this means three or four rather ill-defined fractions were obtained. All of the fractions were yellow in colour, some strongly, others only slightly pigmented.

Ultraviolet-absorption spectra. Each fraction was taken to dryness, yielding a very small quantity of gum, redissolved in absolute ethanol and the absorption spectra determined using a Beckman spectrophotometer. All fractions showed much end absorption but inflexions were observed at 270 mμ. or 256 mμ.

Extraction of dentine with butanolic potassium hydroxide

In order to minimize contamination of the materials with impurities from the charcoal it was decided to omit the adsorption stage and use a more volatile extracting solvent which could be removed by distillation. The solvent chosen was *n*-butanol. Dentine (60 g.) was refluxed for 6 hr. with *n*-butanol containing 5% KOH. This treatment did not remove the organic matter as completely as did alkaline ethylene glycol; the residue after extraction still contained about 0·3% N. The supernatant liquid was separated from the dentine residue, neutralized and taken to dryness on a boiling-water bath under reduced pressure. The residue was extracted with methanol and insoluble non-fluorescent material removed by centrifuging. The methanol solution was again taken to dryness and extracted with 92% (v/v) ethanol; this gave a deep-yellow solution strongly fluorescing blue, the solids of which contained 9·8% N.

Adsorption chromatography. The fluorescent ethanolic solution was poured on to a column of activated alumina and the chromatogram developed as follows: (*a*) *Development with 92% ethanol.* Fraction *A* ran straight through the column and did not appear as a band. It was fractionated on another column into two components A_1 and A_2 by developing with absolute ethanol and 50% (v/v) aqueous ethanol. Fraction *B*, a small distinct fluorescent band. Fraction *C*, a long straggling fluorescent fraction. (*b*) *Development with 10% (v/v) ammonia (sp.gr. 0·88) in ethanol.* Fraction *D*, a distinct band which appeared to develop from a clear stationary yellow band. (*c*) *Development with 25% (v/v) ammonia in ethanol.* Fraction *E*, a large fraction appearing as a yellow band on the column. (*d*) *Development with water.* A large fraction was obtained, brown in colour, rather arbitrarily divided into fractions *F* and *G*.

Each of these fractions (*A–G*) was taken to dryness and redissolved in absolute ethanol. In all cases, particularly so in *E–G*, white, alcohol-insoluble, non-fluorescent solids were removed by this treatment. These solids were ninhydrin-positive.

Acetylation of fractions. Each fraction was again taken to dryness and taken up in a few ml. of pyridine, insoluble non-fluorescent matter being filtered off. An equal volume of acetic anhydride was added and the solutions left for 24 hr. Each solution was taken to dryness several times from absolute ethanol to remove the last traces of pyridine and acetic anhydride. Each fraction was now dissolved in $CHCl_3$ and filtered, where necessary, from the very small insoluble residue.

These $CHCl_3$ solutions of the acetylated fractions were then used for chromatography on activated alumina. After acetylation, fractions A_1, A_2, *B*, etc., were named A_1X, A_2X, *BX*, etc. Each fraction was adsorbed on an alumina column, developed first with $CHCl_3$ then with ethanol and, if necessary, with dilute aqueous ammonia. In all, some forty-eight individual fractions were obtained and the ultraviolet-absorption spectrum of each was determined. Many of the fractions exhibited end absorption only which will not be described further.

Fractions were numbered serially, e.g. fraction EX_4 is the fourth fraction obtained when the column containing EX was developed, $EX_{4.4}$ is the first fraction when EX_4 was re-chromatographed. All the fractions obtained fluoresced and most of them were coloured yellow. Only those which exhibited marked selective absorption in the ultraviolet are described below:

A_1X_8. A very small fraction removed by developing with water; $\lambda_{max.}$ 272 mμ., $\lambda_{min.}$ 230 mμ.

A_2X_1. A small fraction running straight through in $CHCl_3$; $\lambda_{max.}$ 258 mμ., $\lambda_{min.}$ 234 mμ.

A_2X_2. A distinct yellow band developed with $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 232 mμ.

A_2X_4. A small fraction developed with ammonia; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ below 220 mμ.

CX_4. A yellow ethanol-developed fraction; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 230 mμ.

EX_{5A}. A small pale-yellow fraction running straight through in $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 233 mμ.

EX_{5C}. Ethanol fraction; $\lambda_{max.}$ 265 mμ., $\lambda_{min.}$ 230 mμ.

FX_3. A 'tailing' fraction developed with $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 230 mμ.

FX_{5B}. A small fraction developed with ethanol; $\lambda_{max.}$ 265 mμ., $\lambda_{min.}$ 238 mμ.

GX_3. A small fraction developed with $CHCl_3$; $\lambda_{max.}$ 268 mμ., $\lambda_{min.}$ 235 mμ.

GX_{4A}. A small fraction running through in $CHCl_3$; $\lambda_{max.}$ 258 mμ., $\lambda_{min.}$ 227 mμ.

GX_{5A}. A small fraction running through in $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 230 mμ.

The remaining fractions either had end absorption only or slight inflexions in the region of 255 or 270 mμ.

Since the amount of material present in each fraction was so small we were not at this stage able to determine even rough $E^{1\%}_{1\,cm.}$ values.

Absorption between 300 *and* 450 *mμ.* Since the radiation exciting the fluorescence is in the region of 366 mμ., absorption must be occurring near this wavelength. Concentrations which showed selective absorption at 256 or 270 mμ. showed only continuous absorption at longer wavelengths. The fractions were concentrated as much as possible, and the region 300–450 mμ. carefully examined for selective absorption but without success. All fractions showed considerable continuous absorption in this region. We can only conclude that the absorption of the active material is completely masked by the presence in our extracts of relatively vast amounts of impurities.

Tests on the fluorescent extract of dentine. The neutralized butanolic KOH digest of dentine was taken to dryness, extracted with absolute ethanol and the non-fluorescing residue discarded. This orange-yellow solution was evaporated, the residue dissolved in water and subjected to the following tests. When sodium dithionite ($Na_2S_2O_4$) was added the fluorescence, but not the pigment, disappeared; it was regenerated on shaking in air or by adding a few drops of H_2O_2 solution. Acid $KMnO_4$ or bromine water destroyed the fluorescence irreversibly. The addition of H_2O_2 did not appear to affect the fluorescence. (It should be mentioned that freshly voided human urine fluoresced moderately strongly with a blue light when irradiated. The fluorescence behaved in exactly the same way, when treated with the above reagents, as did the dentine extract.)

Acetylation of the dentine extract. Treatment of the extract (after removing the solvent) with pyridine and acetic anhydride rendered it partially soluble in ether. This ethereal solution was bright yellow in colour and fluoresced blue-green. It was separated by chromatography on alumina into a major and two minor components. The major fraction fluoresced blue in ether but on evaporating and redissolving in water the fluorescence was bright yellow. This fluorescence was destroyed by sodium dithionite and the pigment changed from yellow to orange; shaking in air restored the fluorescence and the yellow colour. The yellow fluorescence was very similar to that of riboflavin, but when the latter was treated with pyridine and acetic anhydride, evaporated and extracted with ether, the resulting solution still fluoresced bright yellow.

The resistance of the fluorescing substances to acid digestion. When carrying out N determinations by the Kjeldahl method on the original extracts it was noted that after digestion with H_2SO_4 the blue fluorescence was not completely destroyed.

Acid hydrolysis of the alkaline ethylene glycol extract or the butanolic extract with 6N-HCl for 8 hr. did not destroy the fluorescence. With 6N-HCl in a sealed tube at 100°, the fluorescence was destroyed completely.

Fluorescent material remaining after sulphuric acid digestion. After digestion with conc. H_2SO_4, the digest was diluted with water and neutralized with excess barium carbonate, the mixture being stirred vigorously for 30 min. and then filtered. The filtrate was taken to dryness and extracted with absolute ethanol. This ethanolic solution fluoresced with a blue-violet colour; the absorption spectra had $\lambda_{max.}$ 256 mμ., $\lambda_{min.}$ 228 mμ.; $E^{1\%}_{1\,cm.}$ on our crude material was 138.

It is possible that, during the digestion, condensation reactions have occurred resulting in the formation of fluorescent compounds not present in the original teeth. Nevertheless, it is of interest to pursue this aspect and to try and determine the nature of the reactants.

Extraction of powdered dentine with diethyl ether. About 20 g. of powdered dentine were dried at 100° and continuously extracted with ether for 6 hr. The resulting solution of dentine lipids had only a very faint fluorescence, and the fluorescence of the dentine powder appeared unchanged.

DISCUSSION

Our experiments so far are of an exploratory nature and the results descriptive. We have shown that alkaline ethylene glycol and alkaline *n*-butanol digests of powdered dentine contain fluorescent substances. The dentine residue after such treatment has lost much but not all of its fluorescence. We are satisfied that at least some of these substances are organic in nature since they can be acetylated and methylated and rendered soluble in chloroform. It would also appear that fluorescence is not directly associated with the amino-acids of dentine. Some fluorescing material is present after prolonged heating with concentrated sulphuric acid. Our extracts contained many fluorescent fractions, but these may well be fission products of the active material existing in the dentine.

Blue fluorescent substances occur in many tissues, notably the skeleton, the eye, skin, hair, nails and in the urine. Pirie & Simpson (1946) isolated a substance showing blue fluorescence from the

choroid of the dogfish eye; chemical and spectroscopic evidence make it probable that this substance is xanthopterin. Pterins have also been shown to occur in urine, and uropterin has been proved to be identical with xanthopterin (Koschara, 1943). Although many of our extracts were yellow in colour we have no evidence to suggest that they contain xanthopterin. The ultraviolet-absorption spectrum of this pterin in neutral solution has maxima at 275 and 375 mμ. (Totter, 1944). Some of our fractions had a maximum in the region of 270 mμ., but on no occasion so far has absorption at 375 mμ. been encountered. Goodwin & Srisukh (1950) isolated a yellow pigment from locust eyes which they identified as a pterin closely resembling xanthopterin. This compound absorbed selectively in the region 270–280 mμ. with only a slight band at longer wavelength.

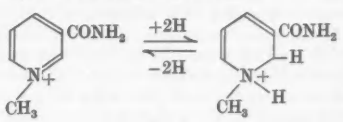
The information we can obtain from spectroscopic examination of our very impure fractions is scanty, and in view of the findings of Goodwin & Srisukh (1950) it is possible that a peak of low intensity is hidden by much irrelevant absorption. Even so, the intensity of the 375 mμ. band of xanthopterin is 0·27 of that of the 270 mμ. band, and we feel that with a ratio of this order we should have observed absorption at the longer wavelength if the 270 mμ. band of our extracts was indeed a component of a pterin spectrum.

Also the fact that the florescence shown by our extracts is unstable to sodium dithionite and potassium permanganate suggests that they do not contain xanthopterin. Becker & Schöpf (1936) report that the fluorescence of pterins is unaffected by these two reagents. Our fractions having selective absorption at 270 mμ. were obtained from the alumina chromatogram at widely separated intervals; most of our fractions had strong end absorption with no selective maxima. It is possible that the substance responsible for the selective absorption may have little to do with the fluorescence. Our activating ultraviolet light was in the region of 366 mμ.; this means that the fluorophoric group must be absorbing in this region. The fact that so far we have not found any selective absorption between 350 and 450 mμ. suggests that the continuously absorbing impurities in our extracts are present in such quantity as to mask completely any selective absorption. The selective absorption at 270 mμ. cannot by itself be responsible for the fluorescence since our light source does not contain radiation at this wavelength. It may be, of course, that the 270 mμ. peak is one component of a spectrum, the longer wavelength of which is hidden by continuously absorbing impurities. This must await further investigation.

One of our acetylated fractions fluoresced bright yellow in aqueous solution, appearing very similar to a solution of riboflavin. It was sensitive to dithionite, but unlike riboflavin the visible pigment did not disappear but changed to orange. In ethereal solution our fraction fluoresces blue; if riboflavin is subjected to acetylation procedures, the ethereal solution still fluoresces yellow. We can only conclude that riboflavin is not responsible for the yellow fluorescence in our extract.

The observation that some fluorescence persists after digestion with concentrated sulphuric acid is of interest, although we realize that it may be due to artifacts. Certain compounds containing nitrogen in the ring, e.g. pyrimidines, are known to be broken down with difficulty by this process. Ring compounds of the pyrimidine series which lack ethylene linkages within the ring may exhibit end absorption but lack any marked degree of selective absorption (Heyroth & Loofbourow, 1934). Pyrimidines may arise in our extracts from the breakdown of nucleoprotein. O'Brien (1944) has reviewed the literature concerning the occurrence in urine of a substance which yields a blue fluorescing compound (F_2) described by Najjar & Wood (1940). Its excretion is increased in proportion to the nicotinic acid intake; it has now been isolated and has a greenish blue fluorescence in alkaline and a blue fluorescence in acid solution (Huff & Perlzweig, 1943; Ellinger & Coulson, 1943; Najjar, White & Scott, 1944). It would appear to be a carbinol derived from *N*-methylnicotinamide. A substance claimed to be identical with the natural product has been prepared from nicotinamide methiodide. *iso*Butanol was used to extract F_2 from urine, and Najjar *et al.* (1944) have suggested that the solvent and the carbinol may form an ether which, they claim, explains the slow increase in fluorescence of *iso*-butanol extracts. We have used alcohols (ethylene glycol and *n*-butanol) for extraction and are aware of the danger of artifact production by similar means.

The substance F_2 must not be confused with the substances present in normal urine which fluoresce without any treatment. F_2 is derived from a non-fluorescent precursor which is rendered fluorescent by making the urine alkaline and extracting with *iso*butanol. It is well known that nicotinamide readily undergoes biological methylation in certain species (including man) and that pyridinium compounds are formed. Such compounds can be reversibly oxidized and reduced.



The reduced or dihydro form in such a system exhibits strong fluorescence when irradiated with

ultraviolet light whilst the oxidized form does not (Morton, 1942). Our extracts behave in exactly the opposite way; the fluorescence is destroyed by reduction with dithionite and restored on reoxidation with air or H_2O_2.

We observed that normal human urine fluoresces blue, and that this fluorescence (not due to F_2) is reversibly destroyed by dithionite, whereas bromine water or acid permanganate causes its irreversible destruction. In addition, according to Ellinger & Coulson (1943), samples of F_2 obtained by them absorbed selectively in the ultraviolet at 271, 306 and 347 mμ. We have therefore no evidence to suggest our extracts contain F_2. There are, however, certain similarities between some fluorescing substance in urine and our extracts which justify further investigation.

Dickson, Forziati & Schoonover (1952) and Dickson, Forziati, Lawson & Schoonover (1952) have utilized the natural fluorescence of teeth in investigating the structure of enamel and dentine. Their photomicrographs show that the intensity of fluorescence appears to be an indication of the relative organic content of the various areas of the tooth. In dentine they report that fluorescence is concentrated along the dentinal tubules and the dentino-enamel junction. The fluorescent materials in our extracts are undoubtedly breakdown products of the organic material existing in the tooth. We are less certain that the mineral phase is devoid of fluorescence for, after repeated extractions with alkaline ethylene glycol, the mineral residue still fluoresced slightly; this may of course be due to traces of organic material not extracted by our treatment.

Reeder & Nelson (1940) have studied the fluorescence associated with proteins. When examined in ultraviolet light of wavelength 310–410 mμ. they found that many proteins gave a bluish white fluorescence in the solid state and a more green fluorescence in solution. Acid hydrolysis increased the fluorescence of those proteins containing tryptophan much more than in the case of those deficient in this amino-acid. Tyrosine and tryptophan were the only amino-acids which gave blue-green fluorescence with glucose when boiled with dilute hydrochloric acid. We are now investigating the fluorescence of various proteins and comparing it with that of the teeth and tooth extracts.

The preliminary investigations reported here have merely cleared the ground a little and have enabled us to define the problem in more adequate terms. We can conclude that the substances responsible for the fluorescence in our extracts are organic compounds apparently present in very small amounts. They contain, or are closely associated with, groups capable of acetylation or methylation. It is of interest to have found some evidence of substances which can undergo reversible oxidation and reduction, but until we have obtained more information we cannot speculate on the nature of any oxidation-reduction system. There must also be a group present which absorbs in the region of the wavelength of the activating radiation (about 366 mμ.). So far we have been unable to detect any selective absorption at this longer wavelength owing to the presence of continuous absorption by the impurities in our extracts. Irradiation with monochromatic ultraviolet light of shorter wavelength may of course reveal additional fluorophoric groups. Our next task is to obtain the fluorescent extracts in a state of greater purity. Although we have isolated many fluorescing fractions, we cannot ignore the possibility that we may be dealing with the same fluorophoric grouping attached to a variety of carriers, which would account for the differing chromatographic behaviour of our samples. The apparent complexity of the problem may therefore be due to our own experimental methods.

SUMMARY

1. Digestion with alkaline ethylene glycol or alkaline *n*-butanol extracts much of the fluorescent material from dentine, that is it is removed along with the organic fraction.

2. By the use of adsorption chromatography on alumina columns many fluorescent fractions have been obtained.

3. Most of the fractions do not exhibit any selective absorption in the ultraviolet region.

4. Some fluorescent material is present in the extract after sulphuric acid digestion.

5. The fluorescence of the dentine extract is due in part to organic substances other than amino-acids.

6. We have some evidence that our extracts contain a fluorescent pigment which can undergo reversible oxidation-reduction, but no evidence that xanthopterin, riboflavin or nicotinamide derivatives are present.

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Chondroitin Sulphuric Acid as a Factor in the Stability of Tendon

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The intercellular substances of connective tissue consist of fibrillar and amorphous components. On morphological grounds the histologists have postulated the presence of an amorphous substance cementing collagen fibrils together into fibre bundles, and an amorphous ground substance in which these bundles are embedded (Maximow & Bloom, 1948). Meyer and his co-workers (Meyer & Smyth, 1937; Meyer & Chaffee, 1941) have isolated the mucopolysaccharides chondroitin sulphuric acid (CSA) and hyaluronic acid from skin, cartilage and tendon, and these substances have been identified with the cement and ground substances, respectively. However, apart from the work of Meyer, Palmer & Smyth (1937) on protein-CSA complexes, of Partridge (1948) on cartilage and of Einbinder & Schubert (1951) on the binding of CSA by collagen, little information is available concerning the association of the mucopolysaccharides and collagen.

Rat-tail tendon can be dissolved in dilute acetic acid (Nageotte, 1927), and from the solution fibres can be reconstituted by dialysis and salt precipitation (Huzella, 1932; v. Buzagh, 1942), and by addition of mucopolysaccharides (Highberger, Gross & Schmitt, 1951). When examined under the electron microscope fibrils precipitated with 0·7–1·5% (w/v) potassium chloride showed the characteristic 640 A. spacing (Bahr, 1950). At these concentrations, needle-shaped crystals or tactoids were produced, having the striated structure of collagen, while outside this range (0·5–10%) long, non-striated, fibrils were formed (Vanamee & Porter, 1951). Mucopolysaccharide-precipitated fibrils were found to have a wide spacing of up to 2000 A., although small concentrations of serum mucoprotein gave the 640 A. spacing (Highberger *et al.* 1951). This was also shown for heparin-precipitated fibrils (Morrione, 1952). However, CSA-precipitated fibrils were either non-striated or had spacings up to 2000 A. (Highberger *et al.* 1951).

In these studies little emphasis was placed on obtaining mucopolysaccharide-free collagen, the tissue being usually extracted with 10% (w/v) sodium chloride or 30% potassium chloride. Partridge (1948) and Woodin (1952) showed that extraction with 10% (w/v) calcium chloride was necessary to remove all the mucopolysaccharide, and this method involved the solution of protein that appears to be of collagenous origin.

Before studying the relationship of mucopolysaccharide to collagen, it seemed essential to obtain mucopolysaccharide-free collagen by other methods. It has been reported (Meyer, Chaffee, Hobby & Dawson, 1941; Madinaveitia & Stacey, 1944) that

testicular hyaluronidase is active against CSA, the mucopolysaccharide component of tendon. It is probable that the same enzyme is active against both hyaluronic acid and CSA (Meyer *et al.* 1941; Humphrey, 1946), but only CSA occurs in tendon. Incubation of tendon with hyaluronidase caused gross changes which were visible to the naked eye to occur, the tendon becoming swollen and frayed, splitting up longitudinally into smaller units. It was decided, therefore, to study this phenomenon in detail. A preliminary communication on part of this work has already been made (Jackson, 1952).

EXPERIMENTAL

Material and methods

Tendon preparation. Fresh calf tendo Achillis was freed from gross fat and loose connective tissue, minced and then cut up finely with scissors, defatted with ethanol and ether, washed with 10% (w/v) NaCl and distilled water, dehydrated in acetone and dried *in vacuo*.

Enzyme preparation. Crude testicular hyaluronidase was prepared by the method of Madinaveitia (1941). This preparation was tested against CSA extracted from bovine trachea (Einbinder & Schubert, 1950) by measuring the liberation of reducing sugars. The relative activity of this preparation compared with that of a standard was not determined.

Shrinkage temperature. This was carried out by placing single fibres in water in a boiling tube fitted with a thermometer. The boiling tube was placed in a water bath, the temperature raised slowly and shrinkage temperature noted.

Total nitrogen. This was determined by the micro-Kjeldahl technique using the distillation apparatus of Markham (1942), digestion being carried out with SeO_2 as catalyst.

Dissolution of tendon

Treatment with hyaluronidase. Tendon (1 g./100 ml. of buffer) was incubated at 37° with hyaluronidase (2 mg./ml.) in 0·1 M-sodium acetate buffer at pH 5·2 containing 0·15 M-NaCl (*A*) (Meyer, 1951). A further portion of tendon was incubated in the same solution with the enzyme omitted (*B*). The material was incubated for 72 hr., incubating solutions being changed every 24 hr. The tendon was filtered off, washed with saline and distilled water, dehydrated with acetone and dried *in vacuo*.

Dissolution by acetic acid. A 1 g. sample of each of *A* and *B* was shaken with 100 ml. 0·4% (v/v) acetic acid for 24 hr., centrifuged, and the supernatant decanted. The extraction was repeated six times, the extracts being collected separately. The residue was washed and dried as before and weighed. Total N was determined on each extract.

Dissolution of reconstituted fibres

Fibres were reconstituted from a solution of enzyme-treated tendon by two methods: (1) by addition of an equal volume of 10% (w/v) NaCl (Huzella, 1932); (2) by addition of an equal volume of CSA (1 mg./ml.) (Highberger *et al.* 1951).

A portion of the CSA-precipitated material was incubated with hyaluronidase as described previously. All three preparations were washed in saline and water, dehydrated with acetone and dried *in vacuo*. A sample (100 mg.) of each preparation was weighed and shaken with 50 ml. 0·4% (v/v) acetic acid for 24 hr., centrifuged, washed and dried as before and reweighed.

RESULTS

Dissolution of tendon after hyaluronidase treatment

During enzyme treatment the tendon fibres became swollen and frayed, and split up longitudinally into smaller units. During the first extraction *A* became considerably more swollen than *B*. The results (Table 1) show that after pretreatment of tendon with hyaluronidase 85% was soluble in dilute acetic acid as compared with 25% of the untreated tendon.

Table 1. *Effect of pre-treatment with hyaluronidase on the extraction of tendon by* 0·4% *(v/v) acetic acid*

(Tendon (1 g.) incubated with 100 ml. of 0·1 M-sodium acetate buffer, pH 5·2, containing 0·15 M-NaCl. Enzyme concentration 1 mg./ml. The figures represent average values of three experiments.)

Tendon	Initial weight (g.)	Residue weight (g.)	Dissolved (% initial weight)
Untreated	1·026	0·769	25·2
Enzyme-treated	1·016	0·153	84·9

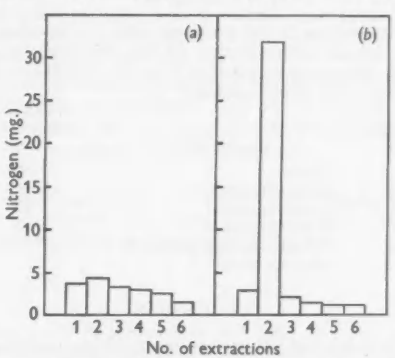

Fig. 1. Total nitrogen of 0·4% (v/v) acetic acid extracts of (*a*) native tendon, (*b*) tendon treated with hyaluronidase.

Determination of total nitrogen of the extracts (Fig. 1) showed that most of it was made soluble during the first two incubations. The low nitrogen content of the first extract of *A* was due to the considerable swelling which took place, 85 ml. of the extracting solution being imbibed by the tendon.

Dissolution of reconstituted fibres

The results (Table 2) show that almost the whole of the salt-precipitated collagen and of the enzyme-treated CSA-precipitated collagen was redissolved by 0·4% (v/v) acetic acid, while CSA-precipitated collagen was virtually insoluble.

Table 2. *Dissolution by 0·4 % (v/v) acetic acid of fibres reconstituted from solution in 0·4 % (v/v) acetic acid by different treatments*

(Samples (100 mg.) shaken with 50 ml. of acetic acid. Fibres in Exp. 3 incubated with 50 ml. of acetate buffer, pH 5·2, containing 0·15 M-NaCl. Enzyme concentration 1 mg./ml. The figures represent average values of three experiments.)

Exp. no.	Type of reconstitution of fibre	Initial weight (mg.)	Residue weight (mg.)	Dissolved (% initial weight)
1	Salt precipitation	103	5	95·1
2	CSA precipitation	110	106	2·7
3	CSA precipitation followed by incubation with hyaluronidase	105	1	99·0

Shrinkage temperature

Native tendon shrinks at 67°. On enzyme treatment the shrinkage temperature was found to be 53°. It was also observed that removal of CSA from CSA-precipitated fibres reduced the shrinkage temperature from 55 to 47°. The lowest shrinkage temperature found was that of salt-precipitated fibres (43°). The results (Table 3) show that absence of CSA or its degradation by hyaluronidase, depresses the temperature at which collagenous fibres shrink.

Table 3. *Shrinkage temperature of various fibre preparations*

(Preparations 2 and 5 treated with hyaluronidase in 0·1 M-sodium acetate buffer, pH 5·2, containing 0·15 M-NaCl. Enzyme concentration 1 mg./ml. Results are the average of six determinations.)

Exp. no.	Type of fibre	Shrinkage temperature (°)
1	Native	67
2	Enzyme-treated	53
3	Salt-precipitated	43
4	CSA-precipitated	55
5	CSA-precipitated and then enzyme-treated	47

DISCUSSION

The swelling of collagenous material such as tendon and skin is considered to be the result of the excess osmotic pressure (swelling pressure) in the protein phase, arising from the difference in concentration of diffusible ions in the protein and external phase, equilibrium being reached when the osmotic pressure is balanced by the cohesive forces of the protein (Procter & Wilson, 1916).

The enzyme treatment of tendon was carried out at pH 5·2. At this pH minimum swelling would be expected; since the swelling pressure is the same in each case, and the enzyme-treated tendon (*A*) was more swollen than the untreated tendon (*B*), it appears that enzyme treatment has decreased the cohesive forces. This increased swelling of *A* was also noted during the first extraction with 0·4 % (v/v) acetic acid. The increased solubility of tendon in dilute acetic acid after enzyme treatment suggests that the swelling pressure has overcome the cohesive forces, causing breakdown of tendon structure. This breakdown of structure was only partial with untreated tendon, but was almost complete with the enzyme-treated material, presumably, because this treatment, as suggested above, has greatly reduced the cohesive forces. The results of the experiments with reconstituted fibres lead to the same conclusion, since absence or removal of CSA resulted in almost complete re-solution.

The shrinkage temperature has been shown to be a measure of the structural stability of collagen expressed in temperature units (Theis, 1946). Astbury (1940) has suggested that any interference with the interaction of any inter-chain linkage must inevitably influence the shrinkage temperature. If, therefore, CSA is concerned with inter-chain linkages, removal of CSA from tendon or its absence from reconstituted fibres should result in a reduction of the shrinkage temperature. This was in fact found, and provides further evidence for the thesis that CSA is responsible to some extent for the structural stability of collagenous fibres.

Strong alkali and calcium chloride solutions have been widely used for the extraction of mucopolysaccharides such as CSA (Meyer & Smyth, 1937). Hence treatment with these reagents would, on the evidence presented here, be expected to reduce the stability of collagenous tissues. Partridge (1948) has pointed out that solvents which are good extractors of CSA from cartilage also reduced the shrinkage temperature of rat-tail tendon. Theis (1946) showed that the presence of 2 M-sodium chloride in the external phase reduces the effect of pH on swelling and shrinkage temperature to a minimum except at a pH greater than 10 when swelling is still very prominent and shrinkage temperature falls rapidly. In contrast, the presence of calcium chloride increases swelling and causes a considerable fall in shrinkage temperature. Bowes & Kenten (1950), in a careful study of the swelling of hide, conclude that the rapid increase in swelling above pH 10 is due to a decrease of cohesion, caused mainly by a breakdown of intermolecular linkages. On the basis of the results of

Partridge (1948) they suggested that some of the intermolecular linkage may be due to the mucopolysaccharides present in hide. Indeed, all these experiments lead to the conclusion that conditions which are favourable to the extraction of mucopolysaccharide are associated with a striking reduction in the stability of the tissues studied. It could be argued (Theis, 1946) that this reduction in the very high pH range is due to a pronounced effect of alkali on hydrogen bond structures. This might lead to a concomitant release of bound mucopolysaccharide. However, it is not certain that strong alkali or calcium chloride does in fact break down the hydrogen bonds. Nor would the enzyme treatment described in this paper be expected to have any effect on these bonds; this treatment leads to a reduction in the stability of tendon similar to that demonstrated by the results of the workers discussed above. Thus an alternative interpretation is that CSA is in fact taking part in the intermolecular linking in the collagenous fibres of connective tissue and that its removal leads to a reduction of fibre stability. Partridge (1948) has suggested that CSA is cross-linked to collagen in cartilage, and a similar suggestion concerning corneal mucopolysaccharide has also been put forward (Woodin, 1952).

The results presented in this paper indicate that CSA plays an important part in the stability of tendon, but this tissue contains only a small amount of mucopolysaccharide. It is possible, therefore, that it enters into the structure of tendon at a fairly high level of aggregation of collagen molecules. There is no evidence to show how CSA is incorporated into the tissue structure, but it is probably firmly linked, since drastic chemical treatment is required to extract all the mucopolysaccharide from such tissues as tendon, cartilage and cornea, and these conditions cause solution of protein of collagenous origin (Meyer *et al.* 1937; Partridge, 1948; Woodin, 1952). Considered in this way the structure of tendon can be visualized as bundles of polypeptide chains bound together by inter-chain forces, these bundles being further aggregated by CSA acting as a cement substance.

Tendon is a specialized form of connective tissue, and it may well be that in other connective tissue structures the relationship between collagen and mucopolysaccharide is different. Nevertheless, the evidence presented in this paper underlines the possible importance of mucopolysaccharides for the stability of connective tissue, and indicates the desirability of carefully considering this factor in studies of the pathogenesis of the so-called collagen diseases in which breakdown of connective tissue bundles is a prominent feature.

SUMMARY

1. Treatment of tendon by testicular hyaluronidase was found to lead to a decrease in the stability of tendon as measured by an increase in swelling, by increased solubility in dilute acetic acid and a decrease in shrinkage temperature.

2. Similar experiments with reconstituted collagen produced the same results.

3. On the basis of this evidence and that of other workers, it is concluded that the mucopolysaccharides play an important part in stabilizing the collagenous fibres of connective tissue.

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The Calcium Binding of Human Serum Albumin in Health and Disease

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Martin & Perkins (1950), studying the calcium-binding capacity of human albumins prepared by a variety of methods, found good agreement between samples prepared from fresh material, provided due care had been taken in the fractionation. They drew attention to two exceptions. The one, a sample of albumin purified by repeated recrystallization with the aid of decanol, gave a low value; the other, a specimen of albumin which had been crystallized with the aid of mercury according to the method of Hughes (1947), gave a high value for calcium binding.

These findings raised the possibility that in normal human serum there are at least two species of albumin differing in their capacity to bind calcium.

In the present work albumins prepared from serum drawn from a number of normal healthy persons have been examined to discover to what extent the calcium binding varies from one individual to another and from one age group to another. In addition, albumins have been separated from the serum of a limited number of patients suffering from a variety of disease processes to discover whether or not disease may alter the calcium-binding capacity of the serum albumin.

A brief note of the observation on the calcium-binding capacity of serum albumin isolated from patients suffering from parathyroid tumours has already been reported by Martin & Perkins (1951). The results recorded in this paper are supplementary to those already published.

EXPERIMENTAL

Preparation of serum albumin. Blood was drawn without venous stasis, from fasting subjects at rest, into clean tubes without anticoagulant and allowed to clot. The fresh serum was removed and subjected to fractionation without delay by the cold methanol procedure of Pillemer & Hutchinson (1945). The albumin obtained in solution was dialysed against repeated changes of 0·15 M-NaCl, the temperature never being allowed to rise above 2°. The final solution was transferred to clean screw-topped bottles and freeze dried. The dried material was then redissolved and the calcium binding studied by the method of equilibrium dialysis described by Martin & Perkins (1950).

Analytical procedures. Determinations of pH were made on all reconstituted samples using a glass electrode and a Cambridge potentiometer.

Protein nitrogen concentrations at each stage were estimated by the standard micro-Kjeldahl technique. Albumin/globulin ratios in the original serum were estimated independently, using Na_2SO_4 as the precipitating agent according to the method of Martin & Morris (1949).

The electrophoretic analyses were carried out in phosphate buffer, pH 8·0, ionic strength $I=0{\cdot}2$ using the moving boundary technique of Tiselius (1939).

The ultracentrifugal analyses were carried out in the oil-driven centrifuge of Svedberg in phosphate buffer, pH 8·0, $I=0{\cdot}2$.

Clinical diagnosis. The clinical diagnoses referred to, unless otherwise stated, have been confirmed by autopsy or operation. The clinical course of all patients has been followed to death or for not less than 2 years.

RESULTS

The serum albumin from twenty-nine normal persons whose ages ranged from 2 to 91 years was examined. The individual values are shown in the

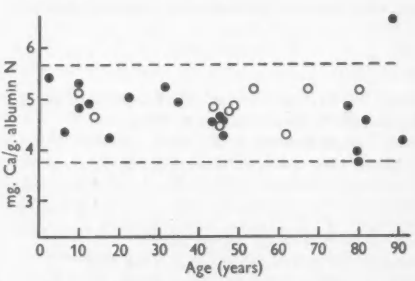

Fig. 1. Values for calcium binding of albumin, isolated from twenty-nine individuals believed to be normal, plotted against age. ●, males; ○, females. Mean value, 4·75. Limits of deviation according to Student's distribution when $P=0{\cdot}01$ are $\pm 0{\cdot}9$. The dotted lines on the figures represent the range of these limits about the mean.

form of a graph in Fig. 1, together with the calculated mean and the limits of variation to be expected in 99 % of persons. The values for the five persons over

80 years show considerable scatter. While every care was taken to exclude organic disease, this was not easy in the oldest patients. In Table 1 are three sets of duplicate estimates, taken at yearly intervals from a single individual, whose detailed life history was known. These values suggest that there are no gross variations from year to year; these and the duplicate analysis of a second subject show the variations in values to be expected from the inherent technical difficulties of the method.

Table 1. *Calcium-binding capacity of normal albumins*

(Albumins separated from the sera collected from two normal individuals on the dates shown. All analyses were carried out between pH 6·4 and 6·8 in 0·15 M-NaCl.)

Initial	Date	Bound Ca (mg./g. albumin N)
N.M.	28. xi. 50	4·4
		4·8
N.M.	7. vi. 51	4·6
		4·4
N.M.	20. x. 52	4·2
		4·5
R.K.	10. vii. 51	5·0
		4·3

Table 2 shows the analysis on eight patients suffering from chronic renal damage and five patients suffering from tumours of the parathyroid gland. The pre- and post-operative levels are shown in three of these patients, together with the post-operative levels on two other patients who have been under observation for 5 and 13 years, respectively. In four of the patients suffering from parathyroid tumours the post-operative percentage renal efficiency, as estimated by the urea clearance technique of Moller, McIntosh & Van Slyke (1928) is shown. A value of 75 % and upward was regarded as normal.

Analyses from six other patients who had been the object of careful clinical study are shown in Table 3. While there is no reason to doubt the diagnosis given in any one of these patients, they form a miscellaneous group, and the levels recorded may or may not be characteristic. They are presented to show that the calcium-binding capacity of serum albumin may be within normal limits in the presence of severe general metabolic disturbance.

Electrophoretic analyses of the albumin obtained by the methanol fractionation from two normal subjects, from two patients suffering from chronic renal damage and from one patient suffering from tumour of the parathyroid are shown in Fig. 2. Ultracentrifugal analyses of two albumins, the one

Table 2. *Calcium-binding capacities of twenty-two human serum albumins separated from thirteen patients in whom the clinical diagnosis was confirmed at autopsy or operation*

(All analyses were carried out between pH 6·3 and 6·9 in 0·15 M-NaCl.)

Patient and date of collection		Diagnosis	Albumin N in serum (g./l.)	N of sample in sac (g./l.)	Donnan coefficient calculated from chloride distribution	Calculated bound Ca inside sac (mg./l.)	Bound Ca (mg./g. albumin N)	Urea clearance Van Slyke method* (%)
B	15. iii. 50	Chronic glomerulonephritis	4·2	2·0	1·02	19·3	9·6	—
B	24. iii. 50		4·3	1·9	1·01	17·1	9·0	—
C	1. vi. 51		3·3	1·9	1·01	17·3	9·1	—
C	12. vii. 51		3·3	2·0	1·02	22·3	11·2	—
D	29. vi. 50		6·7	2·2	1·02	19·3	8·6	—
D	29. vi. 51		4·5	2·76	1·02	57·9	18·8	—
E	24. ix. 52		0·8	0·5	1·003	5·4	12·0	—
F	5. x. 51		3·2	2·75	1·02	32·9	11·9	—
G	29. ii. 52	Chronic pyelonephritis	4·6	1·9	1·01	13·3	7·3	—
H	15. vi. 50	Chronic glomerulonephritis	4·6	2·7	1·01	5·3	2·0	—
H	22. vi. 51		4·2	2·5	1·01	3·3	1·3	—
K	26. ii. 52		3·8	2·6	1·01	5·2	1·9	—
K	31. iii. 52		3·8	1·0	1·01	0·9	0·9	—
N	15. iii. 50	Parathyroid tumour pre-operative	7·4	1·6	1·02	10·7	6·7	—
O	12. vi. 51		6·4	2·4	1·01	21·8	9·0	—
P	17. v. 50		6·5	3·1	1·03	19·3	6·2	—
N	12. vii. 51	Parathyroid tumour post-operative	7·3	4·6	1·03	32·2	7·1	—
O	29. iii. 52		6·4	2·4	1·04	44·4	14·3	30
P	31. v. 50		6·5	3·0	1·04	14·8	4·9	—
P	28. v. 51		6·5	3·0	1·02	14·6	4·9	80
Q	11. v. 51		6·4	2·7	1·02	11·8	4·4	85
R	30. v. 50		4·8	2·7	1·03	17·0	6·3	50

* 75 % upward = normal clearance values by this method.

Table 3. *Calcium-binding capacities of six human serum albumins separated from six patients in whom the diagnosis was established by careful clinical assessment*

(All analyses were carried out between pH 6·4 and 6·6 in 0·15 M-NaCl.)

Patient and date of collection	Diagnosis	Albumin N in serum (g./l.)	N of sample in sac (g./l.)	Donnan coefficient calculated from chloride distribution	Calculated bound Ca inside sac (mg./l.)	Bound Ca (mg./g. albumin N)
T.A. 20. vi. 50	Hypoparathyroid (post-operative)	6·4	3·0	1·03	5·4	1·8
C.H. 22. viii. 50	Hyperthyroid	6·0	3·1	1·02	14·3	4·6
G.R. 31. i. 51	Hypothyroid	5·8	2·9	1·02	12·8	4·4
R.O. 28. iii. 51	Acromegalic	5·8	3·1	1·03	14·5	4·6
L.A. 24. iv. 50	Multiple fractures	6·5	2·9	1·02	11·6	4·0
M.C. 2. ii. 50	Hypertensive	5·6	1·7	1·02	7·2	4·2

from a patient suffering from chronic renal damage and the other from a patient with a parathyroid

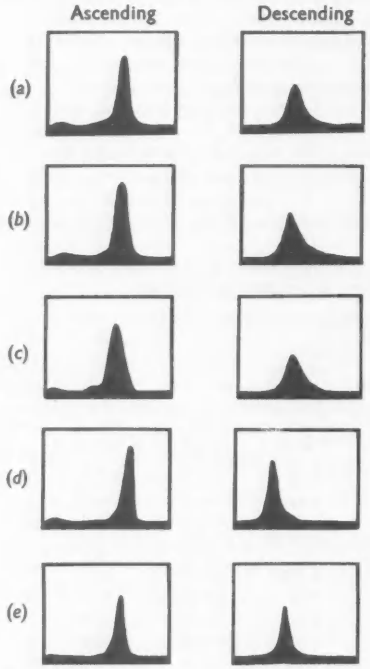

Fig. 2. Electrophoretic analysis of five albumins separated from sera by methanol fractionation and dialysis against 0·15 M-NaCl. The analyses were carried out at 1% (w/v) protein concentration in phosphate buffer, pH 8·0, $I = 0{\cdot}2$. (*a*), normal subject; (*b*), normal subject; (*c*), patient *C*, 12. vii. 51, nephritic; (*d*), patient *D*, 29. vi. 51, nephritic; (*e*), patient *N*, 15. iii. 50, parathyroid tumour.

tumour are shown in Fig. 3. No significant difference was demonstrated either in mobility or sedimentation constant of any of the samples.

DISCUSSION

Pillemer & Hutchinson (1945) described a cold-methanol fractionation which separated almost all the serum albumin from the serum globulin at one step. They published a series of analyses which showed that the relation of serum albumin to serum globulin obtained by this method tallied closely with the values obtained by electrophoretic analysis.

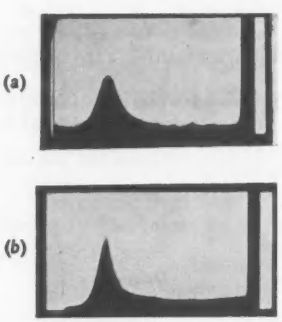

Fig. 3. Ultracentrifugal analysis in phosphate buffer, pH 8·0, $I = 0{\cdot}2$. Field strength 270 000 *g*. Exposures 60 min. after reaching full speed. (*a*), patient *D*, 29. vi. 51; (*b*), patient *P*, 17. v. 50.

Martin & Morris (1949), in a study of methods of estimating the albumin/globulin ratio, confirmed Pillemer & Hutchinson's findings and showed that the concordance held good over a wide range of sera from a variety of conditions in which it is known that there is gross disturbance of albumin/globulin ratio.

Direct electrophoretic analysis of the material obtained by methanol fractionation from normal and pathological sera suggested that it was not less than 94% pure and that contaminating material was α-globulin. Further, calcium-binding studies on the material obtained by the more complicated

techniques of ether and ethanol fractionation gave values that agreed with those obtained by the methanol fractionation.

Leutscher (1940) observed that purified human albumin when examined electrophoretically in acetate buffer at pH 4·0 resolved into two components. His findings have been confirmed in a limited series, but are not presented here in order to avoid suggesting, in any way, that the two components observed are associated with variations in calcium binding.

The original observations on the calcium binding of serum albumin (Martin & Perkins, 1950) suggested that there were at least two varieties of the albumin molecule circulating in normal serum. If that be so, the ratio of the two albumins shows little variation through the normal life span and is materially affected neither by sex nor by the normal period of growth. Further, the limited analyses suggest that there is little variation in the individual from year to year, in conformation with the group survey.

It follows that the deviations from normal observed in patients suffering from advanced renal damage or from the effects of a parathyroid tumour are most probably the consequence of the disease process. Progressive renal damage is well known to be a complication of the activity of a parathyroid tumour (Albright, Aub & Bauer, 1934), while many pathologists have remarked on the hyperplasia of the parathyroids which may be associated with 'primary' renal disease (Gilmour & Martin, 1937; Gilmour, 1947).

The observation that two of the three subjects who continued to have a raised calcium-binding level after operation had continuing renal damage demonstrable by an entirely independent test, while two, who had normal post-operative calcium binding levels, had no demonstrable renal damage by the same test, stresses the close relationship between parathyroid activity and renal function.

Two patients with renal insufficiency yielded albumin with low calcium-binding levels. One of these was the subject of intensive clinical and pathological study over a period of years, and there is some reason to suppose that, terminally, if not earlier in the course of her disease, she may have suffered from hypofunction of the parathyroid. One patient in the miscellaneous group is believed on clinical grounds to be suffering from parathyroid insufficiency. The calcium binding of that patient's albumin was low, by comparison with normals and with the other five patients listed in the miscellaneous group.

It should not be concluded that the observations described are peculiar to albumin or to bivalent ions. Calcium was chosen as the cation to be studied because of its biological importance, albumin because it could be fractionated with the minimum of disturbance and with reasonable purity. The data presented here are no more than a pattern which may have analogies in other disease processes, and with other proteins not yet sufficiently characterized to make reasonably accurate study possible.

SUMMARY

1. The calcium-binding capacity of sixty-two human albumins isolated from forty-eight persons has been examined.

2. The mean binding capacity of albumin separated from normal individuals calculated as mg. calcium/g. albumin nitrogen is $4·7 \pm 0·9$ ($P = 0·01$). There is no significant variation between the ages of 2 and 91 years nor with sex.

3. Of the ten patients suffering from chronic renal damage, eight had significantly raised levels of calcium binding. The level varied with the course of the disease.

4. The albumins from patients with tumours of the parathyroid had raised calcium binding prior to operation. Of the albumins studied from patients after operation three gave normal levels, these being from patients in whom there was no evidence of renal impairment.

We wish to acknowledge the financial help given by the Research Fund of St George's Hospital, the courtesy of the clinicians of St George's in allowing one of us (N.H.M.) to examine their patients, and of Prof. T. Crawford for making his post-mortem records available to us.

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Quantitative Studies of the Avidity of Naturally Occurring Substances for Trace Metals

3. PTERIDINES, RIBOFLAVIN AND PURINES

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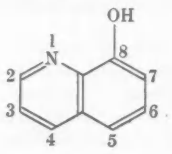
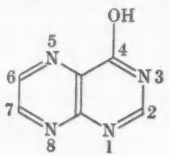
8-Hydroxyquinoline (I) owes its well known avidity for metallic cations to the presence of an ionizable hydroxyl group peri to a tertiary heterocyclic nitrogen atom. The presence of a similar configuration in several of the naturally occurring purines, e.g. guanine (IV), riboflavin (III), and all but one of the naturally occurring pteridines, which are derivatives of (II), gave rise to a suggestion that these substances, also, bind metallic cations (Albert, 1950*b*, *c*). Gibson & Rubbo, 1953). A derivative of 8-hydroxyquinoline (xanthurenic acid) has been found in pathological urine (Musajo, 1935).



(I)



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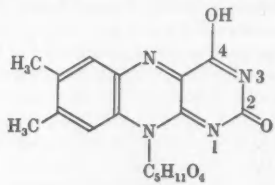

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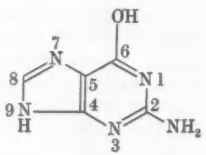

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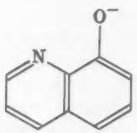

(V)

In the present work the metal-binding properties of these substances have been demonstrated and measured. As a preliminary step, quantitative investigation of the avidity of 8-hydroxyquinoline (I) and its sulphonic acid was undertaken, both because these substances bridge a gap in behaviour that separates the substances to be studied from the amino-acids described in Parts 1 and 2 (Albert, 1950*a*; 1952), and because the stability constants of 8-hydroxyquinoline were required to interpret the antibacterial action of this substance which is under investigation (Albert, Rubbo, Goldacre & Balfour, 1947; Rubbo, Albert & Gibson, 1950; Albert,

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METHODS

All determinations were carried out at 20°. The source of metallic ions was as given in Part 1 (Albert, 1950*a*); the source of each metal-binding substance is given in Table 1, together with its ionization constants (the tolerances are inserted wherever the determination was made specially for the present work). The metal-binding substances used were all chemically pure. Purity was confirmed: (*a*) by the same constant being obtained from all parts of the potentiometric titration curve (in the absence of metals); (*b*) by melting point or by chromatography in three separate solvents when the substance had no melting point; (*c*) by ultimate analysis (nos. 2–12 inclusive and 14).

The titrations were carried out as described in Part 1. One equivalent of 0·1 N-KOH was used except for nos. 2, 5, 6 (which required two equivalents) and 10 (which required three). When metallic ions were present, the titrations were carried out at the concentrations shown in Table 1, except when the precipitation of complexes made a lower concentration necessary. In no case was a concentration lower than 0·001 M used.

Symbols

K_a = Ionization constant.

pK_a = Negative logarithm of K_a.

$[M^{2+}]$ = Concentration of free metallic ions.

[Sc] = The concentration of the complex-forming species, e.g. (V) in the case of 8-hydroxyquinoline (I). It was calculated according to equations (vi) or (vii) of Part 2 (Albert, 1952) according to whether one or two equivalents, respectively, of alkali were used in the titration (see above).

K' (the first partial stability constant)

$$= \frac{[\text{the 1:1 complex, e.g. (VI)}]}{[\text{Sc}]\,[M^{2+}]}. \qquad (a)$$

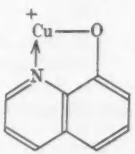
K'' (the second partial stability constant)

$$= \frac{[\text{the 2:1 complex, e.g. (VII)}]}{[\text{Sc}]\,[\text{the 1:1 complex}]}. \qquad (b)$$

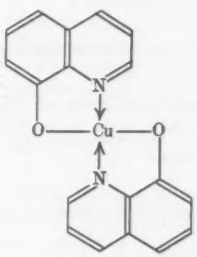
K_s (the overall stability constant, governing the complete reaction)

$$= \frac{[\text{the 2:1 complex, e.g. (VII)}]}{[\text{Sc}]^2\,[M^{2+}]}. \qquad (c)$$

$\bar{n}$ = The average number of molecules of the substance combined with one atom of metal. Values of $\bar{n}$ were calculated according to equation (v) of Part 2 (Albert, 1952).



(VI)



(VII)

Calculations

$$\log K' = \log \bar{n} - \log (1 - \bar{n}) - \log [\text{Sc}]. \qquad (i)$$

$$\log K'' = \log (\bar{n} - 1) - \log (2 - \bar{n}) - \log [\text{Sc}]. \qquad (ii)$$

$$\log K_s = -2 \log [\text{Sc}], \text{ when } \bar{n} = 1{\cdot}00. \qquad (iii)$$

$$\log K_s = \log K' + \log K''. \qquad (iv)$$

$$\log K_s = \log \bar{n} - \log (2 - \bar{n}) - 2 \log [\text{Sc}]. \qquad (v)$$

The choice of the correct equation, from the above, is discussed on pp. 647–8.

Derivation of equations. By definition, $\bar{n}$ = average number of molecules of substance combined with one atom of metal, hence

$$\bar{n} = \frac{[\text{VI}] + 2\,[\text{VII}]}{[\text{VI}] + [\text{VII}] + [M^{2+}]},$$

where [VI] and [VII] refer to the concentrations of the 1:1 and 1:2 complexes as exemplified by formulae (VI) and (VII) respectively. Then

$$\frac{\bar{n}}{2 - \bar{n}} = \frac{[\text{VI}] + 2\,[\text{VII}]}{[\text{VI}] + 2\,[M^{2+}]}.$$

When [VI] is small compared to $[M^{2+}]$ and [VII], then

$$\frac{\bar{n}}{2 - \bar{n}} = \frac{[\text{VII}]}{[M^{2+}]}.$$

Hence from (*a*)

$$K_s = \frac{\bar{n}}{(2 - \bar{n})\,[\text{Sc}]^2}, \text{ whence (v).}$$

Derivations of equations (i) to (iv) have been given (Albert, 1950*a*).

RESULTS

The stability constants are reported in Table 2. These naturally occurring substances were found to form metal complexes less soluble, on the whole, than those of the simple amino-acids discussed in Part 1 (Albert, 1950*a*). Thus, in several cases it was impossible to obtain the second of the partial stability constants (K''). Accordingly, closely related substances which might form more soluble complexes were sought. In this way, investigation of a more extensive portion of the titration curve in an analogous substance could shed further light on the complex-forming properties of the natural substance.

Complex formation by the majority of the substances followed the same sequence as was found for the amino-acids (Albert, 1950*a*, 1952). First, one atom of the metal combined with one molecule of the substance. The 1:1 complex (e.g. VI) thus formed did not appreciably combine with a second molecule of the substance to give the 1:2 complex (e.g. VII) until at least 80% of the 1:1 complex had been formed (i.e. $\bar{n} = 0{\cdot}8$). Normal addition, in this manner, can be diagnosed by the shape of the formation curve obtained when $\bar{n}$ is plotted against $-\log$ [Sc]. In Fig. 1, *C* represents such a curve, theoretically derived,* and *A* shows that it is parallel to a typical experimental curve (that of copper + glycine). This mechanism will be referred to as *stepwise addition*. In all such cases, both equations (i) and (ii) give consistent values (within $\pm 0{\cdot}2$) for $\log K'$ ($\bar{n} = 0{\cdot}1$–$0{\cdot}8$) and $\log K''$ ($\bar{n} = 1{\cdot}2$–$1{\cdot}8$) respectively.

A few of the substances listed in Table 1 showed quite a different pattern of addition to certain metals, particularly to copper. In these exceptional cases the shape of the formation curves (e.g. *B* in Fig. 1) indicated that no appreciable quantity of the 1:1 complex accumulated, even in the first half of

* Derived by adopting any value for K' and working out values of $-\log$ [Sc] for regular increments of $\bar{n}$, from equation (i).

Table 1. *Ionization constants* (20°)

(Sources: *A*, British Drug Houses Ltd. Recrystallized. *B*, Albert, Brown & Cheeseman (1951). *C*, Albert & Brown (1953). *D*, Albert & Wood (1952). *E*, Albert, Brown & Cheeseman (1952*b*). *F*, Brown (1953). *G*, Analytically pure grade, kindly presented by the American Cyanamid Company (Batch SR-9891). *H*, Special sample, free from aliphatic acids of low mol.wt., kindly presented by Roche Products Ltd., Welwyn Garden City. (Found: C, 54·2; H, 5·3; N, 15·0. Calc. for $C_{17}H_{20}O_6N_4$: C, 54·3; H, 5·4; N, 14·9%.) *I*, Nutritional Biochemicals Corporation, Ohio. Recrystallized. *J*, British Chemicals and Biologicals Ltd. Recrystallized. *K*, Levene & Jacobs (1910).)

No.	Substance	Temp. dried at (°)	Source	Concentration (M)	pK_a (acidic)	pK_a (basic)
1	8-Hydroxyquinoline	20	*A*	0·005	9·89 (±0·03)	5·13 (±0·02)
2	8-Hydroxyquinoline-5-sulphonic acid (+$2H_2O$)	20	*A*	0·005	1·3* (approx.) 8·74 (±0·02)	4·15 (±0·02)
	Pteridine					
3	4-Hydroxy-	110	*B*	0·01	7·89	<1·3†
4	2:4-Dihydroxy-	110	*B*	0·01	7·92 >12	<1·3
5	4:6-Dihydroxy- (+$1H_2O$)	110	*C*	0·0013	6·08; 9·73‡	<2
6	4:7-Dihydroxy-	110	*C*	0·001	6·08; 9·62‡	<2
7	2-Amino-4:6-dihydroxy- (+$1H_2O$) (xanthopterin)	110	*D*, *E*	—	6·3; 9·23‡	1·6
8	6-Anilinomethyl-4-hydroxy-	110	*F*	0·001	7·92 (±0·02)	<3
9	6-Anilinomethyl-4-hydroxy-*p*-sulphonate, sodium salt (+$2H_2O$)	20	*F*	0·004	7·91‡ (±0·03)	<2·5
10	Pteroylglutamic acid (+$2H_2O$)	20	*G*	0·002	8·26‡, § (±0·06)	—
11	Riboflavin	110	*H*	0·001	9·93‖ (±0·05)	‖
12	Inosine	20	*I*	0·01	8·82¶ (±0·01)	1·5**
13	Hypoxanthine	110	*J*	0·005	8·94¶ (±0·02) >11·5	1·98 (±0·02)
14	Xanthosine (+$2H_2O$)	20	*K*	0·002	5·67†† (±0·02) >11	<2·5
15	Guanosine (+$1H_2O$)	20	*A*	0·002	9·31‡‡ (±0·07)	‡‡
16	Uric acid	110	*A*	—	§§	—
17	Adenine	110	*A*	0·005	‖‖	4·22 (±0·02)
18	Adenosine (+$1\frac{1}{2}H_2O$)	20	*A*	0·005	—	3·52¶¶ (±0·04)

* 1·3 is from the $-SO_3H$ group (see text).
† This and other upper limits for basic constants were obtained in the strongest solutions which could be made (often supersaturated).
‡ The 4-hydroxyl group.
§ No other pK_a above 5; in the titrations involving metals (Table 2) no pH value below 6·5 was encountered and hence the lower pK_a values do not enter into the calculations.
‖ Michaelis, Schubert & Smythe (1936) obtained 9·8 (and −0·2 for the basic group) spectrometrically.
¶ Ogston (1936) obtained 8·7 (H_2 electrode, 25°) for both substances.
** Approximately.
†† Ogston (1935) obtained 6·0 and 7·7 for xanthine (H_2 electrode, 18°).
‡‡ Levene & Simms (1925) obtained 9·16 (and 1·6 for the basic group). For guanine, Taylor (1948) obtained 9·2 (12·3 for the acidic glyoxaline group and 3·3 for the basic group).
§§ 5·40 and 5·54 (Bernouilli & Loebenstein, 1940).
‖‖ Taylor (1948) obtained 9·80 (and 4·15 for the basic group), H_2 electrode.
¶¶ Ogston (1936) obtained 3·6; Levene & Simms (1925), 3·45.

the titration. Thus this 1:1 complex must be able to compete strongly with the free metallic cations for the unbound complex-forming species. This mechanism will be referred to as 'simultaneous addition'. When it is operating, equations (i) and (ii) no longer give consistent results, and their place must be taken by equation (v). Conversely, equation (v) does not give consistent results in the usual case where very little of the 1:2 complex is formed below $\bar{n} = 0{\cdot}8$. Intermediate behaviour was sometimes encountered; even the best example of rapid consecutive addition (4-hydroxypteridine + copper, curve *B*, Fig. 1) shows a tendency to combine according to equation (i) at the very beginning of the titration, but it then approximates to equation (v) and adheres to it (within experimental error) until the end of the titration.

Equation (iii) for $\log K_s$ is valid regardless of the type of addition taking place, because it is used only when $\bar{n} = 1{\cdot}00$: at this value of $\bar{n}$, all formation curves pass through the same point (see Fig. 1).

DISCUSSION

Quinolines

8-Hydroxyquinoline (I; oxine) proved to have such a high avidity for metals that it was possible to add an equivalent of hydrochloric acid (three equiva-

lents for Cu^{2+} and Fe^{3+}) before commencing the titration with alkali and thus obtain a series of consistent values for log K'. When this addition of acid was omitted, precipitation occurred at the beginning of the titrations, which had therefore to be discarded (the Mg^{2+} complex was more soluble, however). No conditions could be found to obtain any direct values for Zn^{2+} because of solubility difficulties. Values of log K' and log K'' for the 1:1 and 1:2 complexes of ferric ion with oxine were found to be 12·35 and 11·3 respectively. The values for the other metals are reported in Table 2.

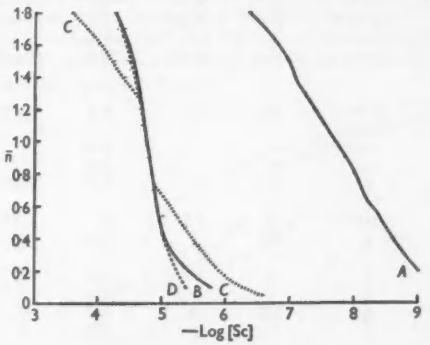


Fig. 1. Formation curves for complexes. *A*, glycine (0·01 M) and Cu^{2+} (0·005 M); *B*, 4-hydroxypteridine (0·01 M) and Cu^{2+} (0·005 M); *C*, theoretical curve for *B*, assuming stepwise addition. Log K' has been taken as about 1 unit larger than log K'' to give normal interaction at $\bar{n} = 1$ (in the absence of this interaction, the upper and lower limbs of this curve would be horizontal at $\bar{n} = 1$, and would not touch one another); *D*, theoretical curve for *B*, assuming simultaneous addition.

Because all the log K' values of 8-hydroxyquinoline are so close to those of its sulphonic acid, it is likely that the log K_s values of the two substances will also be nearly the same. This hypothesis is strongly supported by some titrations of oxine in the presence of metallic ions carried out in aqueous dioxan, a solvent which gives high values because of its low dielectric constant (e.g. log K' for Cu^{2+} is 15·0), but which permits the whole titration to be carried out without precipitation (Johnston & Freiser, 1952; Maley & Mellor, 1949). These titrations in dioxan show that the frequently observed (approximate) relationship between log K' and log K_s (namely $\log K_s = 2 \log K' - 1$) holds for oxine. If this relationship is applied to log K' in Table 2, values of log K_s for 8-hydroxyquinoline are obtained which correspond fairly closely to those of its sulphonic acid.

No precipitation occurred during the titrations of 8-hydroxyquinoline-5-sulphonic acid and the curves were clearly seen to be of type *A* (Fig. 1), that is to say, similar to those given by the amino-acids. Apart from the values in Table 2, the following values were obtained for Fe^{3+} (log $K' = 12$; log $K'' = 11{\cdot}2$; log $K''' = 9{\cdot}4$; hence log $K_s = 36$) and Ca^{2+} (log $K' = 3{\cdot}5$; log $K_s = 5{\cdot}8$). Because of the great avidity of this substance for Cu^{2+} and Fe^{3+}, mixtures with these cations had to be back-titrated with N-hydrochloric acid to obtain log K', because merely mixing the reagents (i.e. without any addition of alkali) produced values of $\bar{n}$ above 1·0.

It is seen from Table 2 that the stability constants for 8-hydroxyquinoline and its sulphonic acid are greater than any that have yet been encountered in this series of papers, except those of cysteine (Albert, 1952). These quinolines, like the amino-acids, show the usual relative avidities for metals (Albert, 1950*a*, 1952), namely,

$$Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+}.$$

Mellor & Maley (1948), who discovered this series, placed Zn^{2+} near Co^{2+}, and Cd^{2+} near Fe^{2+}. Zinc and cadmium, however, belong to an independent series and hence their positions among the above transitional elements is likely to vary a little (Irving & Williams, 1948; Williams, 1950).

Pteridines

4-Hydroxypteridine (II), in spite of its structural similarity to 8-hydroxyquinoline, has a far lower affinity for metallic ions (see Table 2); even compared with glycine its log K values are low. Nevertheless, the low value of the acidic pK_a of 4-hydroxypteridine ensures that it is almost entirely in the form of the complex-forming species (the anion) at the physiological pH of 7·3, a property which would enable it to compete successfully with the common amino-acids in the presence of an equivalent of metallic ions: in fact it would prevail over glycine, proline and many other common amino-acids for Fe^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+} and Mn^{2+} (Albert, 1950*b*). This information was derived from a comparison of $\bar{n}$ values at pH 7·3. However, under conditions where the proportion of complex-forming agents to metal is relatively high, it is more correct to compare the K_s values directly. When this is done, the position of 4-hydroxypteridine becomes less favourable, as can be seen from Table 2. These two different aspects of comparative avidity are referred to below, under pteroylglutamic acid and guanosine.

4-Hydroxypteridine shows two peculiarities. First, the curve for Cu^{2+} is as shown in Fig. 1 (*B*) and hence almost simultaneous addition is taking place (this seems also to be the case with Fe^{2+} and Mn^{2+}, but precipitation prevents inspection of the entire curves). The same results were obtained with copper sulphate as with copper chloride, but copper

nitrate precipitated a NO_3^--containing complex (likewise zinc sulphate precipitated an anion-containing complex at an earlier stage in the titration than zinc chloride). Secondly, the avidity for Fe^{2+}, relative to that for Cu^{2+}, Ni^{2+} and Co^{2+}, is higher than is usually encountered among complex-forming substances (Mellor & Maley, 1948; Irving & Williams, 1948). The cobalt complex, unlike those of the amino-acids (particularly histidine), did not show any appreciable tendency to absorb oxygen.

4-Hydroxy-6:7-dimethylpteridine (Albert, Brown & Cheeseman, 1951) gave almost exactly the same curves and constants as 4-hydroxypteridine. 2:4-Dihydroxypteridine gave similar curves (there was no doubt that the Fe^{2+} curve was like B) but somewhat lower constants (Table 2).

Table 2. *Stability constants of complexes with metallic cations (at 20°)*

Substance	Cu^{2+} log K'	Cu^{2+} log K_s	Ni^{2+} log K'	Ni^{2+} log K_s	Zn^{2+} log K'	Zn^{2+} log K_s	Co^{2+} log K'	Co^{2+} log K_s
Glycine (for comparison)	8·5	15·4*†	6·1	11·0*†	5·2	9·3*†	5·1	8·9*†
8-Hydroxyquinoline	12·2	—	9·9	—	§	—	9·1	—
8-Hydroxyquinoline-5-sulphonic acid	12·5	23·1*†	10·0	18·1*†	8·4	15·1*†	9·2	16·8*†
Pteridine								
4-Hydroxy-	‖	9·5*¶	4·4	7·8* 8·1†	3·3	§	3·8	6·6*†
2:4-Dihydroxy-	‖	8·3¶	3·7	6·5*	—	—	3·2	§
6-Anilinomethyl-4-hydroxy-	—	§	§	—	§	—	3·5	§
6-Anilinomethyl-4-hydroxy-*p*-sulphonate, sodium salt	—	§	4·0	§	3·4	§	3·7	6·5*
Pteroylglutamic acid	§	7·8*	§	9·0*	§	7·5*	§	8·1*
Riboflavin	‖	13·1* 12·9¶	4·1	§	5·6	§	3·9	§
Inosine	5‡	§	3·3	§	§	—	2·6	§
Hypoxanthine	6·2	§	4·7	§	§	—	3·8	§
Guanosine	6‡	§	3·8	§	4·6	§	3·2	§
Xanthosine	3·4	§	3·0	§	2·4	§	2·8	§

Substance	Cd^{2+} log K'	Cd^{2+} log K_s	Fe^{2+} log K'	Fe^{2+} log K_s	Mn^{2+} log K'	Mn^{2+} log K_s	Mg^{2+} log K'	Mg^{2+} log K_s
Glycine (for comparison)	4·5	8·1*†	4·3	7·8*†	3·2	5·5*†	2‡	4‡
8-Hydroxyquinoline	7·2	—	8·0	—	6·8	—	4·5	—
8-Hydroxyquinoline-5-sulphonic acid	7·6	13·5*†	8·4	15·1*†	6·6	11·5*	4·8	8·5*†
Pteridine								
4-Hydroxy-	3·4	§	3·4‡	5·9* 6·2¶	2·4‡	4·5¶	<1	—
2:4-Dihydroxy-	—	—	2·9‡	5·6¶	—	—	—	—
6-Anilinomethyl-4-hydroxy-	§	—	§	—	—	—	—	—
6-Anilinomethyl-4-hydroxy-*p*-sulphonate, sodium salt	—	—	§	—	§	—	—	—
Pteroylglutamic acid	4·0	6·7*	§	7·9*	§	6‡	—	**
Riboflavin	4·7	§	7·1	§	3·4	§	—	—
Inosine	—	—	3‡	§	—	—	—	—
Hypoxanthine	—	—	3·9	§	2·4	§	—	—
Guanosine	4·0	§	4·3	§	3·0	§	—	—
Xanthosine	—	—	<2	—	—	—	—	—

* Calculated from value of log [Sc] at $n = 1{\cdot}00$, by equation (iii).
† By addition of log K' and log K''.
‡ Approximately.
§ Poor solubility of complex prevented determination.
‖ No direct evaluation possible but, for purposes of comparison, may be taken as half log K_s.
¶ From equation (v).
** No depression of pH in presence of Mg^{2+}, hence log K_s is below 6.

Table 3. *Depression of the* pH *of solutions of pteridines* (0·001 M) *in water* (*oxygen free*) *on the addition of metallic salts* (0·0005 M; pH 5·6–6·4)

Pteridine	$CuCl_2$	$Ni(NO_3)_2$	$ZnCl_2$	$Co(NO_3)_2$	$CdSO_4$	$FeSO_4$	$MnSO_4$
4:6-Dihydroxy- (pH 5·01)	1·23	0·88	0·46	0·42	.—	0·43	0
2-Amino-4:6-dihydroxy- (xanthopterin) (pH 5·49)	1·90	1·46	1·00	1·09	0·90	0·57	0·13

4:7-Dihydroxypteridine (Albert & Brown, 1953) differs from this isomeride in that the ionization of the non-chelating hydroxyl group is complete before the 4-hydroxy group has ionized to any extent (see Table 1). Because of the insolubility of its complexes, the only value obtainable was log K' for Co^{2+} (5·7). This value is considerably in excess of those given by the pteridines already discussed, but follows the rule that, in any series, weakening of acidic properties (or strengthening of basic properties) increases the avidity for metals. In the present case, the 4-hydroxyl group is weakened by the usual coulombic effect due to the nearness of another anion (that of the 7-hydroxyl group). From this reasoning, high avidity was expected also from 4:6-dihydroxypteridine and xanthopterin (2-amino-4:6-dihydroxypteridine).

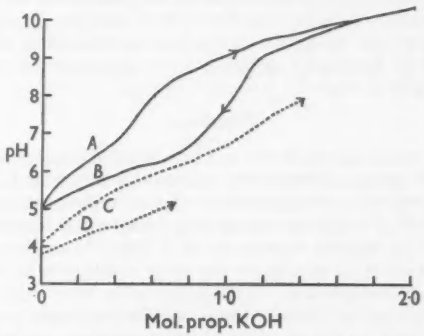
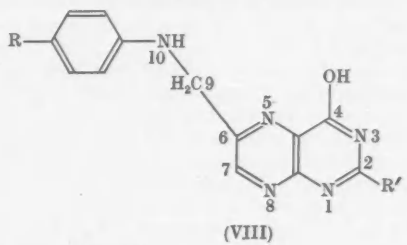


Fig. 2. Titration of 4:6-dihydroxypteridine (0·001 M). *A*, Titration with KOH (0·1 M); *B*, back-titration of *A* with HCl (0·1 M). The noncoincidence of *A* and *B* shows that a slow tautomerization is taking place; *C*, as *A*, but Ni^{2+} is present (0·0005 M); *D*, as *A*, but Cu^{2+} is present (0·0005 M).

As it happens, pteridines bearing a hydroxyl group in the 6-position undergo a slow tautomerization upon titration (Albert, Brown & Cheeseman, 1952*a*). 4:6-Dihydroxypteridine gives the pair of curves *A* and *B* (Fig. 2) upon titration with alkali and acid respectively, the curve *A* tending to approach *B* with the lapse of time. Xanthopterin behaves similarly. It is evident that bivalent cations (as exemplified by nickel and copper in curves *C* and *D* respectively) cause a large displacement of curve *A* to more acid pH values. Unfortunately, stability constants cannot be calculated from this liberation of hydrogen ions unless the solubility of the complex permits titration to proceed well into the region of the second equivalent of alkali (this was possible in the case of Ni^{2+} only, which gave log $K' = 5·5$). This limitation arises from the fact that the lower half of curve *A* corresponds to no pK_a, and owes its horizontal component mainly to the presence of small amounts of mono-anion, for which the lower half of curve *B* provides the true pK_a (trial calculations showed that the amount of mono-anion was varying unpredictably throughout the titrations involving metals). For xanthopterin, which gave even more insoluble complexes, no constant could be recorded. However, the large liberation of hydrogen ions, on bringing together this substance and metallic cations, makes it clear that the avidity for metals was considerably greater than that of 4:6-dihydroxypteridine (see Table 3). This increase in avidity, due to the 2-amino group, is referred to again later, under pteroylglutamic acid.

The inhibition of the oxidation of a colourless precursor of melanin by xanthopterin (Isaka, 1952) may be due to the abstraction of Cu^{2+} (or another heavy cation) by the pteridine at this step which normally occurs in the dark and without enzymes. Many other 4-hydroxypteridines inhibit the initial oxidation of dihydroxyphenylalanine in melanogenesis (Polonovski, Gonnard & Baril, 1951).



(VIII)

(*a*) R and R′ = −H.
(*b*) R = $-SO_3Na$, R′ = −H.
(*c*) R = $-CONH\text{-}CH(COOH).CH_2.CH_2.(COOH)$, R′ = $-NH_2$ (pteroylglutamic acid).

6-Anilinomethyl-4-hydroxypteridine (VIII, *a*) was chosen as a simple model for pteroylglutamic acid (folic acid) (VIII, *c*). It was thought that these substances might use simultaneously both $N_{(5)}$ and $N_{(10)}$ in binding metals, the distances being suitable. Such terdentate complexes would give high values of log K'. Unfortunately, the complexes of (VIII, *a*) are only sparingly soluble and only in the case of Co^{2+} could a significant portion of the titration curve be inspected. To confer greater solubility, a sulphonic group was introduced, giving (VIII, *b*), which revealed more of the chelation process before precipitation of the complexes occurred. Neither in the case of (VIII, *a*) nor (VIII, *b*) did the magnitudes of the constants suggest any departure from the mode of chelation of 4-hydroxypteridine. It is regrettable that the Fe^{2+} complexes of (VIII, *a* and *b*) were so insoluble, because this metal (which has a greater tendency to hexacovalence than any of the others studied) could most readily employ the extra co-ordination latent in (VIII) and thus achieve terdentate combination, as opposed to

the more common bidentate combination shown in (VII).

Pteroylglutamic acid (VIII, *c*) forms highly insoluble salts with the heavy metals, but only in the lower pH range. This effect would seem to be due to ordinary salt formation between the carboxylic acid groups and the metallic cations. Above pH 7, where the ionization of the 4-hydroxyl group becomes substantial, these precipitates dissolve and the colours characteristic of 4-hydroxypteridine complexes appear, for example the yellow precipitate of ferrous pteroylglutamate turns into a blood-red solution as the metal is transferred from a carboxyl group to the pteridine nucleus, to give complexes of the types (VI) and (VII). Hence it was found that the best way to carry out these titrations was to dissolve the pteroylglutamic acid (0·002 M) with the aid of from 2·0 to 2·4 equivalents of potassium hydroxide, then to add the metallic salt and to proceed with the alkaline titration. This method revealed a good deal of the titration curves (from $\bar{n} = 0{\cdot}7$ to 1·8), including the important point where $\bar{n} = 1{\cdot}0$. The results, given in Table 2, show that the constants are, in almost every case, higher than those of any pteridine which it has been possible to measure so far. This heightened avidity is most likely due to the presence of the 2-amino group (as in the pair xanthopterin and 4:6-dihydroxypteridine discussed above). The high constants of the pteroylglutamic acid cobalt and iron complexes, coupled with the high degree of ionization ($pK_a = 8{\cdot}3$) of the hydroxyl group in pteroylglutamic acid combine to make it a powerful competitor for these two metals, even in the presence of amino-acids. The Mellor-Maley sequence (see p. 649) is not strictly adhered to, the principal change being a strong depression of the constant for Cu^{2+} (compare the log K_s values for pteroylglutamic acid with those for 4-hydroxypteridine, Table 2). The cause of this depression may well be a certain amount of steric hindrance in the 1 : 2 complex between the side chain and the oxygen in the 4-position of the other pteridine molecule, an effect that will be easily visualized from an inspection of (VII). The only ion used in this work that would be affected by such a hindrance, is Cu^{2+}, because it alone forms exclusively planar complexes. If this hypothesis is correct, log K' should be normal. This cannot be verified because of insolubility, but the first half of the titration curve of 6-anilinomethyl-4-hydroxypteridine appeared to be normal. Except for cadmium, the titration curves of the other metals with pteroylglutamic acid, as far as they can be followed in either direction, seem to have a fairly steep slope (i.e. a tendency towards simultaneous addition is present). No evidence for terdentate combination was found for any metal. That the benzoylglutamic acid portion does not take part in complex formation, at or above pH 7, follows from the investigation of L-benzoylglutamic acid in Part 2 (Albert, 1952). Pteroylglutamic acid did not appear to form any complex with Fe^{3+}. The dimethyl ester (in which the α- and γ-carboxylic groups of the glutamic acid portion are esterified) proved to be too insoluble to titrate.

The following pteridines, which are too sparingly soluble to titrate, showed evidence of chelation by giving a red colour with ferrous sulphate in borate buffer at pH 8·0: 4:6:7-trihydroxy-, 2-amino-4-hydroxy- and 2-amino-4:7-dihydroxy-pteridine (the last named is *iso*xanthopterin). This colour reaction is also given by all the pteridines of Table 2. 2-Amino-4:6:7-trihydroxypteridine (leucopterin) was too insoluble at pH 8 to be tested. 2-, 6- and 7-Hydroxypteridines showed no evidence of combining with metallic ions. When 4-aminopteridine hydrochloride was titrated in the presence of Cu^{2+}, a weak tendency (log $K' = 2{\cdot}5$) to complex formation was revealed. Complexes of this kind are given by nearly all amines (cf. ammonia + Cu^{2+}, log $K' = 4{\cdot}0$).

Riboflavin

Although riboflavin has been studied extensively for the last 20 years, its considerable metal-binding powers have only recently been described (Albert, 1950*c*). This is not surprising, because the formula is not usually written as (III), but as a tautomer which does not show the close analogy with 8-hydroxyquinoline. Titrations gave the results reported in Table 2. Only Cu^{2+} showed more consistent results when calculated from (v) than from (i), and hence it may be supposed that this metal allows simultaneous addition (see p. 648). This evidence of lack of steric hindrance (contrast with pteroylglutamic acid, above) may spring from the non-planarity of the *iso*alloxazine nucleus, which is not conjugated throughout.

The most remarkable feature of the log K' values for riboflavin is the high value obtained for the Fe^{2+} complex. Apparently, the avidity of riboflavin for ferrous ions is greater than for any other metallic ion studied. Unfortunately the titration of Fe^{2+} could be followed only as far as $\bar{n} = 0{\cdot}30$ before precipitation of the complex occurred, so that nothing is known about the shape of the curve except that there was no evidence of simultaneous addition. Riboflavin must be added to the very short list of known substances (e.g. *o*-phenanthroline; 2:2′-dipyridyl) which promote Fe^{2+} out of its natural position in the avidity sequence of ions (see p. 649; also under guanosine, below).

It is interesting to compare the log K' values of riboflavin, glycine and 8-hydroxyquinoline because all three substances have the same acid ionization constant ($pK_a = 9{\cdot}9$), and hence their relative avidities under all circumstances are proportional to

their K' values. It will be seen that riboflavin has more avidity than glycine for several metals, particularly for Fe^{2+} where the avidity is nearly as great as that of 8-hydroxyquinoline. Riboflavin did not appear to combine with Fe^{3+}; moreover, it did not appear to be reduced by Fe^{2+}.

These results open up the possibility that the activity of some riboflavin-containing enzymes may be associated with metallic ions. It is hoped that those who work with the yellow enzymes will report any inhibitions obtained with complex-forming agents (e.g. 8-hydroxyquinoline). In this respect, xanthine oxidase would be interesting, because its substrate (xanthine), its product (uric acid), its coenzyme (a riboflavin derivative) and 2-amino-6-formyl-4-hydroxypteridine which inhibits it very strongly (Lowry, Bessey & Crawford, 1949) can all form complexes, like (VI), with a bivalent cation. It is interesting that riboflavin has already been found associated with iron, namely in a protein (conalbumin) which is found in the avian blood stream and in egg-white (Bain & Deutsch, 1948).

Purines

It is surprising that the metal-binding properties of purines having a free hydroxyl group in the 6-position have remained largely unsuspected. Their avidity (see Table 2) is comparable to that of the pteridines (other than pteroylglutamic acid). A tendency for the value for Fe^{2+} to exceed that of Co^{2+} (and even Ni^{2+}), a feature reminiscent of riboflavin, is seen most strongly in guanosine. All the purine complexes seemed to be formed normally, i.e. by stepwise (as opposed to simultaneous) addition. The choice of the right constants for the calculations presented no difficulty in the case of inosine and guanosine, which have only one acidic group. In the case of xanthosine and hypoxanthine, which have a second, weakly acidic, group of $pK_a > 11$, the more consistent results were obtained by assuming that the pK_a constants 5·7 and 8·9 (respectively) belonged to the complex-forming 6-hydroxyl group. As in the pteridines, an amino group in the 2-position raises the constants, whereas a similarly placed hydroxyl group lowers them.

The figures obtained for guanosine are particularly interesting because this purine occurs in nucleic acids. It is seen to have the same avidity for Fe^{2+} as glycine has (as measured by $\log K'$); indeed its pK_a of 9·3 (as against 9·9 for glycine) would make it slightly the more effective competitor under certain circumstances (as discussed above for 4-hydroxypteridine). Some titrations were also performed on uric acid, for which the order of avidity was plainly $Fe^{2+} > Cu^{2+} > Co^{2+}$, as shown by depression of pH. Because the titration was carried out in supersaturated solutions, and because of the difficulty of assigning pK_a values to the correct acidic groups, no calculations were attempted for these uric acid complexes.

Adenosine (which has no acidic group) forms no detectable complex with Cu^{2+}, Co^{2+} or other metal, the same pH values being obtained on titration in the presence or absence of the metallic cation. Adenine gave a weak complex with copper ($\log K' = 2·5$) which is more likely to be of the type given by many other amines with this metal, rather than one involving the acidic group in the 9-position. Whether adenine (0·005 M) was titrated in the absence or presence of Co^{2+} (0·0025 M), the same pH values were obtained, thus indicating that any complex formed with cobalt can have little stability. Liquier-Milward (1951) has isolated a 1:1 cobalt-adenine complex from aqueous solution, but the above titration shows that the avidity of the adenine for Co^{2+} is very low.

Colours of complexes

The natural amino-acids form blue complexes with Cu^{2+}, green with Ni^{2+} and Fe^{2+} and pink with Co^{2+}. The only exceptions seem to be cysteine (red with Ni^{2+}, Fe^{2+} and Co^{2+}) and histidine (which has both orange and pink Co^{2+} complexes). The purine complexes are similar in colour to those of the amino-acids. The complexes of riboflavin are yellow. The pteridines (including pteroylglutamic acid) and the 8-hydroxyquinolines form green complexes with Cu^{2+} and Ni^{2+}, orange with Co^{2+} and red with Fe^{2+}. The quinolines form dark-green complexes with Fe^{3+}, whereas the other substances mentioned seem not to form complexes with this cation.

SUMMARY

1. The avidities of natural and synthetic pteridines, of riboflavin and of several natural purines for the ions of heavy metals have been measured and recorded in the form of stability constants. For purposes of comparison, 8-hydroxyquinoline and its sulphonic acid were also investigated.

2. These substances form a related series in so far as they combine with metals through the same arrangement of atoms, namely, a hydroxyl group peri to a ring-nitrogen atom. Two different patterns for the stages of combination were demonstrated.

3. Riboflavin and guanosine showed a heightened affinity for Fe^{2+}, and pteroylglutamic acid showed a lowered affinity for Cu^{2+} (relative to the usual gradation of affinity for the different metals). Thus this series shows evidence of specificity for particular metallic cations which is entirely lacking among the amino-acids.

4. Some quantitative implications for conditions in the living cell are discussed.

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Some Observations on the Instability of Adenine Nucleotides in Bird-liver Preparations

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In the course of a study of purine metabolism in suspensions of bird liver it was observed that incubation in the presence of 2:4-dinitrophenol (DNP) results in a considerable formation of free purines. It seemed unlikely that these would be of synthetic origin, and an investigation of possible catabolic sources showed that the liberated purines could be accounted for almost entirely by an extensive breakdown of the free adenine nucleotides in the tissue. The nucleic acid content of such preparations remained relatively unaffected. Other inhibitors of oxidative phosphorylation and, furthermore, the addition of magnesium ions gave similar results. Investigations with the soluble and insoluble fractions of the suspensions and with chick-liver slices are also described.

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MATERIALS AND METHODS

Chicks. Day-old chicks were obtained from hatcheries and fed on chick mash, which was gradually supplemented with, and finally replaced by, crushed oats and greenstuffs at 8 weeks. Chicks were used from 2–12 weeks old. They were usually allowed to feed until shortly before being killed, but in two experiments (88 and 90) they were starved for 20 hr. before death.

Pigeons. Adult pigeons were obtained from dealers and used at once.

Uric acid. Uric acid was determined by the colorimetric method of Brown (1945), modified as described by Dresel & Moyle (1950).

Pentose. Pentose was determined by the method of Mejbaum (1939) with modifications suggested by Ogur & Rosen (1950).

Inorganic, 10 min. acid-labile and total phosphates. These were determined by the method of Fiske & Subbarow (1925), using the micromodifications of Mitchell & Moyle (1951).

Nucleic acids. Nucleic acids were determined essentially by the method of Schmidt & Thannhauser (1945), modified as follows: the preliminary extraction of the trichloroacetic acid precipitate with lipid solvents was omitted, and the ribonucleic acid (RNA) estimated by ultraviolet absorption at 260 mμ., using the molar extinction coefficient of 10 000 for an average tetranucleotide (Wyatt, 1950; Mitchell & Moyle, 1951).

Hypoxanthine. Hypoxanthine was determined enzymically by the method of Kalckar (1947).

Inosine and inosinic acid. These were determined collectively after conversion to hypoxanthine with 0·5N-H_2SO_4 (Kalckar, 1947).

Adenosine monophosphate (5-AMP), Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), and yeast adenylic acid. These were determined spectrophotometrically, using an adenylic acid deaminase (preparation *A*, Kalckar, 1947), a pyrophosphatase from potato and an adenosine deaminase and phosphatase preparation from intestinal mucosa (Kalckar, 1947). The last was found to contain some xanthine oxidase activity which had to be allowed for in the present work. Furthermore, it appears that this preparation will also estimate certain dinucleotides containing adenylic acid, such as, for example, diphosphopyridine nucleotide.

Derivatives of guanine. These were determined collectively as hypoxanthine after acid hydrolysis and treatment with a preparation containing nucleoside phosphorylase and guanase activity (Kalckar, 1947).

Diphosphopyridine nucleotide. This was determined spectrophotometrically with a lactic dehydrogenase preparation.

Spectrophotometric determinations were carried out with a Beckman model DU spectrophotometer.

Preparation of suspensions. The bird was decapitated and bled, the liver excised, and, after removal of the gall bladder, cooled rapidly to 0° in saline. A suitable portion was weighed, quickly chopped on a cold tile and then ground up, usually with 1·5 vol. ice-cold 0·13M-KCl–0·03M-potassium phosphate buffer, pH 7·4, in a Potter-Elvehjem glass homogenizer for 1–2 min. Such a preparation was essentially free from cells other than erythrocytes. The suspension was strained through muslin, and samples pipetted into flasks which already contained all other constituents for incubation.

Preparation of soluble (S) *and insoluble* (I) *fractions*: the suspension was centrifuged at 2000*g* for 15 min. The supernatant was decanted and made up to the volume of the original suspension with 0·13M-KCl–0·03M-potassium phosphate buffer, pH 7·4 (*S*). The residue was washed twice with 0·9% KCl in the cold and then made up to the same volume with the above KCl-phosphate mixture (*I*); this preparation is similar to the cyclophorase preparation of Green, Loomis & Auerbach (1948).

Incubations were carried out in air except where otherwise stated.

2:4-Dinitrophenol (DNP). This was recrystallized from water and used at a concentration of $1·8 \times 10^{-4}$M throughout.

RESULTS

The additional uric acid formed on incubation of chick-liver suspensions in the presence of DNP often amounted to more than 3 times that formed in the control flasks and reached values up to 0·7 mg./g. wet weight liver. DNP caused no rise in uric acid content if the proteins of the suspension were precipitated prior to incubation, nor if the system was kept at 0° instead of being incubated at higher temperatures.

Effect of DNP on tissue nucleic acids. With the experimental conditions used, the breakdown of nucleic acids during incubation of the tissue alone amounted to 2–18%. The additional breakdown of ribonucleic acids (RNA) due to DNP was 6–9% in one experiment and in all others not more than 2%. The deoxyribonucleic acids (DNA) were probably unchanged, the apparent increases being due to artifact (see below). For comparative purposes it is assumed that nucleic acids are composed of tetranucleotides, each of which would give rise to two molecules of free purine. The results summarized in Table 1 show that the losses of RNA due to DNP were very much less than would be required to account for the gain in free purine.

The apparent small increase in RNA in some experiments led to the suspicion that DNP itself might contribute to the ultraviolet absorption by being carried down with the original protein precipitate and then being redissolved in N-NaOH during the Schmidt & Thannhauser procedure. A determination of the absorption at 360 mμ. of the neutralized RNA extract (Table 1, Exp. 96) showed that some 20% of the added DNP was present in

Table 1. *Effect of DNP on nucleic acid content of the liver suspension*

(Samples of suspension incubated with 0·13M-KCl containing 0·03M-potassium phosphate buffer, pH 7·4, except in Exp. 96 where phosphate buffer was 0·012M and flasks also contained 0·016M-$KHCO_3$; gas phase 95% O_2+5% CO_2 in Exp. 96 and air in all others. Time of incubation 15 min. in Exps. 85, 86 and 88, and 30 min. in Exps. 59, 95 and 96. Temp. 38°.)

Exp. no.	Wet wt. liver in flask (mg./ml.)	Total RNA + DNA after incubation without DNP	Changes due to incubation with DNP		
			RNA	DNA	Free purine
		(μmoles purine/g. wet wt.)			
59 (chick)	50	15·4	−0·20	+0·19	+2·98
85 (chick)	148	14·9	−0·26	−0·08	+0·42
86 (chick)	169	14·2	+0·08	+0·03	+0·62
88 (chick)	85	15·3	−0·09	+0·10	+1·72
95 (chick)	56	17·2	−1·07	+1·24	+4·29
95 (chick)	167	17·0	−0·77	+0·17	+3·29
96 (pigeon)	116	18·1	+0·29	+0·39	+2·85

Table 2. *Effect of DNP on adenine nucleotides of the liver suspension*

(For conditions of incubation see Table 1. Substrates were added to Exp. 90 (0·02 M-α-ketoglutarate) and Exp. 92 (0·01 M-glutamate). Time of incubation 15 min. in Exps. 88, and 92, and 30 min. in Exps. 90, 95 and 96.)

Exp. no.	Wet wt. liver in flask (mg./ml.)	AMP, ADP and ATP after incubation (μmoles adenine/g. wet wt.) 0°	38°	38° + DNP	Adenine lost due to DNP (%)	Purine gained due to DNP: Uric acid (μmoles/g. wet wt.)	Hypoxanthine	Purine gained accounted for as adenine lost (%)
88 (chick)	85	2·00	1·82	0·38	79	1·66	—	87
90 (chick)	150	1·29	0·86	0·28	68	0·48	0·62	53
95 (chick)	55	—	4·58	0·67	85	4·29	0·00	91
92 (pigeon)	116	2·79	2·82	2·23	21	—	0·33*	179
96 (pigeon)	116	—	3·27	0·74	77	—	2·30*	110

* These figures include both free hypoxanthine and hypoxanthine in the form of inosine and inosinic acid.

this fraction, and it was calculated that this accounted exactly for the apparent 2 % gain in RNA. The losses in RNA shown in Table 1 are therefore a little low, but the error incurred is too small to affect the conclusions materially.

The apparent gain in DNA, which, in some cases, corresponded rather closely to the loss in RNA, is most probably an artifact. It seems conceivable that the DNP carried down with the precipitate of nucleic acids inhibits the hydrolysis of RNA, some of which is therefore reprecipitated with the DNA. Alternatively, the DNP carried down with the protein may be in the form of a phosphate derivative, thus estimating as DNA.

Effect of DNP on nucleotide content of the suspension. Although, in the intact tissue, adenine mononucleotides may be present largely in the form of adenosine polyphosphates (Davidson & Waymouth, 1944; Rapoport & Nelson, 1945; see also Table 5), it seems that dispersal causes a very considerable loss of pyrophosphate bonds, 80–100 % of the adenine mononucleotides being recovered as 5-AMP, even in samples not incubated at 38°. In most cases incubation at 38° brought about very little change in the ratio of AMP to ADP and ATP, with the exception of Exp. 92 (Table 2), where the adenosine polyphosphate content rose from 20 to 49 % during incubation, the total adenine mononucleotide content remaining essentially unchanged.

Table 2 shows that incubation in the presence of DNP results in a very considerable loss of adenine mononucleotides, amounting, in the case of the chick, to 68–85 % of the nucleotides normally recovered after 15–30 min. incubation. An attempt to correlate these losses with the corresponding gain in free purines was not entirely satisfactory, but the figures appear to be of a similar order and suggest that the adenine mononucleotides should be considered as the chief source of the additional purine liberated in the presence of DNP.

Purine derivatives other than those discussed above would also be present in suspensions, for instance nucleotides resulting from nucleic acid breakdown and coenzymes. It was found that endogenous yeast adenylic acid (2- or 3-AMP), guanylic acid and diphosphopyridine nucleotide were not broken down to a significantly greater extent in the presence of DNP. In the case of diphosphopyridine nucleotide, most of that present initially was lost even on incubation without DNP. Other coenzymes, if affected, would probably be present in insufficient amounts to add appreciably to the total purine liberated.

Effect of incubation of the suspension on composition of insoluble fraction. Samples of a potassium chloride suspension were incubated 30 min. at 0° or 38° both with and without DNP. They were then centrifuged and washed according to Green's procedure for the cyclophorase preparation (Green *et al.* 1948). The residue (R_2L) was taken up in 0·9 % potassium chloride and the protein precipitated with perchloric acid, the final concentration of acid being 3 % (w/v). The supernatant should contain nucleotides and cofactors which had remained attached to the insoluble fraction of the suspension during incubation and washing. The ultraviolet absorption at 260 mμ., pentose, inorganic, 10 min. acid-labile, and total phosphates were estimated. The results of several experiments are summarized in Fig. 1. Incubation at 38° in the presence of DNP led to a considerable loss of phosphorus-containing components from the insoluble fraction. Molar ratios indicated that a large part of these were probably of nucleotide structure. At 0°, DNP removed material estimating as inorganic P (Teply, 1949) and some 10 min. acid-labile P, but no whole nucleotides.

Nucleotide content of the soluble and insoluble fractions of the suspension after separate incubation. Table 3 illustrates the results of an experiment where the effect of DNP on the complete suspension (*H*), the insoluble fraction (*I*) and the soluble fraction (*S*) were studied side by side. Samples of each preparation were incubated 30 min. at (*a*) 0°, (*b*) 38° and (*c*) 38° in the presence of DNP.

α-Ketoglutarate was added throughout in order to allow for the removal of endogenous substrates from (*I*). After incubation, samples (*a*), (*b*) and (*c*) from (*H*) and (*I*) were centrifuged and washed with 0·9 % potassium chloride, and the nucleotide content of the insoluble and supernatant fractions analysed separately after precipitation of the proteins with perchloric acid. It was found that the effect of DNP in suspensions is not reproducible in the separated soluble and insoluble fractions. Degradative changes similar to those caused by DNP in the suspension were observed in the soluble fraction on incubation alone, and DNP caused very little, if any, additional breakdown. Incubation of the insoluble fraction alone resulted in a 75 % loss of attached nucleotides, but these were recovered, apparently unchanged, in the supernatant obtained after recentrifuging. In fact, the recovery of adenine nucleotides in the supernatant was 160 % of the nucleotides lost from the insoluble material. Part of the surplus nucleotide probably resulted from nucleic acid breakdown.

Thus, although the enzymes responsible for degradative changes of free nucleotides are absent from the insoluble fraction, the nucleotides are more easily dislodged when the latter is incubated alone than when incubated as the suspension. It would be of interest to study the effect of added adenine nucleotides in such preparations.

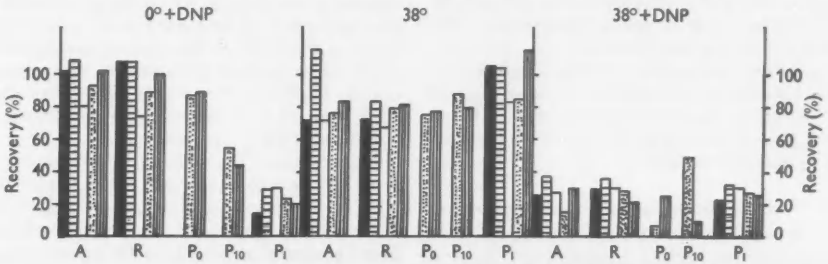

Fig. 1. Fate of perchloric-acid-soluble components attached to insoluble fraction after incubation of suspension at 38° and treatment with DNP. For experimental technique see text. Recoveries expressed as percentage of amounts found after incubation at 0° without DNP. A = ultraviolet absorption at 260 mμ.; R = pentose; P_0 = organic P – 10 min. acid-labile P; P_{10} = 10 min. acid-labile P; P_I = inorganic P. ■, ▤, □, ▨, ▥, correspond to results from five experiments.

Effect of other inhibitors of oxidative phosphorylation. Changes in the purine-containing components of chick-liver suspensions, similar to those reported above, were caused by other inhibitors of oxidative phosphorylation. Thus, a liberation of uric acid occurred after incubation with azide (0·017 M), calcium chloride (0·005 M) and cyanide (0·001 M). Arsenite (0·009 M) gave rise to an accumulation of hypoxanthine rather than uric acid, owing

Table 3. *Effect of incubation with and without DNP on adenine nucleotide content of the soluble and insoluble fractions of a chick-liver suspension*

(For conditions of incubation see Table 2, Exp. 90. Time of incubation 15 min. For preparation of fractions see methods.)

	Suspension			Insoluble fraction			Soluble fraction		
	0°	38°	38° + DNP	0°	38°	38° + DNP	0°	38°	38° + DNP
Perchloric acid extracts from supernatants									
5-AMP, ADP and ATP (μmoles)	0·82	0·55	0·16	0·06	0·21	0·07	0·70	0·07	0·11
Adenine estimated with adenosine deaminase (μmoles)	0·11	0·02	0·13	0·10	0·24	0·28	0·14	0·24	0·07
Total adenine nucleotides	0·93	0·57	0·29	0·16	0·45	0·35	0·84	0·31	0·18
Perchloric acid extracts from insoluble material									
5-AMP, ADP and ATP (μmoles)	0·16	0·10	0·05	0·14	0·04	0·03	—	—	—
Adenine estimated with adenosine deaminase (μmoles)	0·21	0·11	0·01	0·10	0·02	0·00	—	—	—
Total adenine nucleotides	0·37	0·21	0·06	0·24	0·06	0·03	—	—	—
Change in adenine nucleotides (μmoles)		−0·52	−0·95	—	+0·11	−0·02	—	−0·53	−0·66
Change in free purines (μmoles uric acid and hypoxanthine)		+0·47	+1·28	—	+0·03	+0·10	—	+0·81	+0·82

to the inhibition of xanthine oxidase by this reagent (Barry, Bunbury & Kennaway, 1928). However, 0·02 M-sodium sulphate, which, according to Lehninger (1949), is a potent uncoupling agent of oxidative phosphorylation, did not increase uric acid formation or inhibit oxidative phosphorylation in the present system. Finally, anaerobiosis alone was found to be as effective as DNP in stimulating the breakdown of adenine nucleotides in the suspension. It seems, then, that impairment of oxidative phosphorylation, however this may be effected, will lead to an increased breakdown of free nucleotides in the suspension.

Effect of magnesium. The experiment illustrated in Table 4 shows that incubation of the suspension with magnesium chloride (0·005 M) results in an extensive breakdown of adenine nucleotides, similar to that obtained with DNP.

Table 4. *Comparison of effects of magnesium ions and DNP on adenine nucleotides of the suspension*

(For conditions of incubation see Table 1, Exp. 88; concentration of magnesium ions was 0·005 M.)

Additions	None	DNP	$MgCl_2$
Adenine as AMP, ADP and ATP (μmoles)	1·55	0·32	0·44
Uric acid (μmoles)	0·83	2·25	1·78
Additional uric acid accounted for as adenine lost (%)	—	87	117

Inhibition of oxidative phosphorylation in chick-liver slices. The effect of anaerobiosis and of treatment with DNP on the nucleotide content of chick-liver slices is illustrated in Table 5. Although, as was usually the case with suspensions, incubation of the tissue alone resulted in the loss of some adenine nucleotides, this was much increased during anaerobiosis as well as by the addition of DNP. The colorimetric method of determining uric acid was insufficiently sensitive to decide conclusively whether the adenine nucleotides were broken down completely to free purines.

DISCUSSION

It is hoped that the present investigation has served to emphasize the instability of bird-liver adenine nucleotides. A very extensive loss of easily hydrolysable phosphate occurs on grinding of the tissue alone, but a more complete degradation of adenine nucleotides is observed after incubation of suspensions or slices in the absence of active phosphorylating processes. The nucleic acids appear to be relatively more stable, for, under conditions where the addition of DNP resulted in the complete breakdown of 60–80 % of the adenine nucleotides, the breakdown of nucleic acids was scarcely accelerated. LePage (1949) also noted only a slow loss of nucleic acids from rat-liver suspensions when no system for maintaining ATP was present.

The enzymes responsible for the degradation of 5-AMP appear to be confined to the soluble fraction of the suspension, as Potter, Lyle & Schneider (1951) also concluded. Since oxidative phosphorylation is carried out chiefly in the mitochondria, it is not surprising that the factors tending to restrain adenine nucleotide breakdown were found to be associated with the insoluble fraction. However, the regulative mechanism appears to be complex. In most experiments the endogenous nucleotides, although present largely in the form of 5-AMP, were relatively resistant to breakdown during incubation unless inhibitors of oxidative phosphorylation were added. *In vitro*, there appears to be little uptake of radioactive phosphate into 5-AMP as opposed to ATP and flavin adenine dinucleotide (Hummel & Lindberg, 1949; Good & Leaf, 1950), and it seems that the loss of 5-AMP cannot therefore be attributed, simply, to a removal of synthetic activity.

The present work may have little bearing on studies pertaining to the action of DNP on insoluble and mitochondrial preparations, since no significant changes in the nucleotide content of such preparations were observed.

Table 5. *Effect of DNP and of anaerobiosis on the nucleotide content of chick-liver slices*

(300–350 mg. wet weight slices were incubated 30 min. at 37° in Krebs-Ringer phosphate containing 0·2 % glucose. After incubation they were dropped into 10 % (w/v) perchloric acid and ground up rapidly in a small, loosely fitting, glass homogenizer. After centrifuging the supernatant was decanted and the residue washed once with 1 % perchloric acid. The combined supernatants were neutralized and the adenine nucleotide content determined.)

Exp. no.		Slices precipitated at start	Incubation		
			Aerobic	Aerobic plus DNP	Anaerobic
			(μmoles/g. wet wt.)		
89	5-AMP	0·43	0·25	0·59	—
	ADP and ATP	1·20	1·03	0·32	—
	5-AMP, ADP and ATP	1·63	1·28	0·91	—
91	5-AMP	0·50	0·27	0·50	0·45
	ADP and ATP	1·03	1·03	0·27	0·30
	5-AMP, ADP and ATP	1·53	1·30	0·77	0·75

The acceleration of adenine nucleotide breakdown by magnesium ions is of particular interest. Although alkaline phosphatases are activated by magnesium ions (Roche & Nguyen-van Thoai, 1950), the rate of breakdown of 5-AMP in rabbit-liver extracts is not stimulated by magnesium ions (Conway & Cooke, 1939). Swanson (1951) has demonstrated a stimulation of adenosine triphosphatase in rat-liver suspensions by 0·005 M-magnesium, and the action of magnesium in unmasking adenosine triphosphatase activity in mitochondria (Potter & Simonson, 1952) may also be significant. Thus magnesium ions, like inhibitors of oxidative phosphorylation, may stimulate nucleotide breakdown by reducing the level of easily hydrolysable phosphate. This does not, however, explain why Swanson did not also observe an increased rate of breakdown of 5-AMP, as would be expected in the light of the present work. This discrepancy may be due to species difference.

The stability of adenine nucleotides in tissue preparations has not been studied extensively, but observations similar to the above have been made by other workers. A ready deamination of 5-AMP in muscle breis in the absence of phosphorylating processes was demonstrated by Ostern & Mann (1935). Eiler & Allen (1938) showed that liberation of adenosine from purine nucleotides in rabbit blood occurs in the absence of glycolysis. Jeener (1943) observed an extensive loss of adenine nucleotides from liver slices of the rabbit, rat and pigeon during anaerobiosis. Finally, Rapoport (1945) has pointed out that fasting of rats alone might lead to appreciable losses in the adenine nucleotide of the liver. A loss in adenine nucleotides in the absence of phosphorylating processes thus seems to be fairly universal, but the extent of the breakdown may vary from tissue to tissue and also with the type of preparation used.

SUMMARY

1. Incubation of potassium chloride suspensions of bird livers with 2:4-dinitrophenol results in the formation of free purines such as hypoxanthine and uric acid.

2. Most of the liberated purine is derived from the adenine nucleotides of the suspension, which are, in part, attached to the particles. No significant increase in nucleic acid breakdown was noted.

3. Other inhibitors of oxidative phosphorylation, such as azide, cyanide, arsenite, calcium ions and anaerobiosis, exert a similar effect, and so do magnesium ions.

4. Incubation of the soluble fraction of the suspension alone results in a rapid breakdown of adenine nucleotides, whereas incubation of the particles alone results in a dislodgement of attached nucleotides, but no breakdown, even in the presence of 2:4-dinitrophenol.

5. Similar effects are obtained with chick-liver slices, incubated anaerobically or in the presence of 2:4-dinitrophenol.

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The Specificity of Pigeon Brain Aceto-cholinesterase*

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Earlier work by the author and coworkers (Adams & Whittaker, 1948; Adams, 1949; Whittaker, 1949; Mounter & Whittaker, 1950; Mounter, 1951) has shown that the so-called 'true' or 'specific' cholinesterases of erythrocytes, brain and cobra venom, previously believed to act only on acetylcholine and a few other esters, are in reality capable of hydrolysing a wide range of non-choline esters, 3:3-dimethylbutyl acetate,

$$(CH_3)_3C.CH_2.CH_2.O.CO.CH_3$$

the carbon analogue of acetylcholine,

$$(CH_3)_3N^+.CH_2.CH_2.O.CO.CH_3,$$

being the most rapidly hydrolysed of all non-choline aliphatic esters so far examined (for literature see Whittaker, 1951).

The work on brain cholinesterase has only been briefly reported (Whittaker, 1949). In this paper a fuller account of the specificity of pigeon-brain cholinesterase is presented, together with a few additional experiments on the localization of the enzyme in avian brain. Pigeon brain was selected on account of its high cholinesterase activity (nearly four times that of mammalian brain). It was shown by Adams & Thompson (1948) to resemble erythrocyte cholinesterase in its choline ester specificity. It has now been found to possess essentially the same pattern of specificity towards aliphatic esters as other aceto-cholinesterases (Sturge & Whittaker, 1950).

METHODS

Substrates and inhibitors. 3:3-Dimethylbutanol was prepared according to Birch (1949), with the following modifications. Ethyl cyano*iso*propylideneacetate was prepared according to Wideqvist (1949). Ethyl 2-cyano-3:3-dimethylbutyrate from the Grignard reaction was converted in one stage to *iso*propylmalonic acid by refluxing for 3 hr. with equal proportions by volume of H_2SO_4, acetic acid and water; after decarboxylation, the product was reduced to 3:3-dimethylbutanol with lithium aluminium hydride (Nystrom & Brown, 1947). Unsuccessful attempts were made to prepare the alcohol from di*iso*butylene (cf. Homeyer, Whitmore & Wallingford, 1933) and pinacone (cf. Cavalieri, Patterson & Carmack, 1945). The other substrates have been described by Sturge & Whittaker (1950).

Measurement of enzyme activity. This was performed manometrically, using Ammon's (1933) modification of the Warburg technique with the precautions and controls previously described (Sturge & Whittaker, 1950). 100 μmoles of aliphatic esters and 30 μmoles of acetyl-β-methylcholine (β-acetoxypropyltrimethylammonium chloride) were used per ml. Enzyme activity is expressed as amount of acid formed at s.t.p. in μl./mg. dry wt./hr. (Q value), in μl./ml./hr. or as a percentage of the acetyl-β-methylcholine (MCh) rate under identical conditions of pH, temperature and enzyme concentration.

Preparation of enzyme. Most tissues possessing cholinesterase activity contain one or more additional enzymes capable of hydrolysing aliphatic esters (ali-esterases) (Richter & Croft, 1942). Pigeon-brain homogenates were found to hydrolyse tributyrin and *n*-amyl butyrate at appreciable rates, substrates which are not appreciably attacked by the purified cholinesterase of red cells (Mendel & Rudney, 1943; Mounter & Whittaker, 1950). It was therefore suspected that pigeon brain also contained an ali-esterase, and attempts were made to free the cholinesterase from it. The course of the purification was followed by means of these two substrates and by *iso*amyl acetate which is split by both ali-esterase and cholinesterase.

The final method of purification was as follows. A pigeon was decapitated, the brain (including cerebellum) dissected out, the blood blotted off with filter paper, and the tissue homogenized for 2 min. in 20 ml. water in a Folley type homogenizer. A sample was withdrawn for testing (Q(MCh), 89). The remainder (15 ml.) was centrifuged on a bench angle centrifuge for 10 min. at 2000 *g*. The precipitate was resuspended in water (8 ml.) and again centrifuged. Only about 7% of the original activity now remained in the precipitate. The supernatant and washings were centrifuged at 16000 *g*. for 30 min. in an Ecco Ultima II centrifuge with stainless steel cups. The precipitate was resuspended in water (6 ml.) and again collected at 16000 *g*. for 30 min. This process was repeated. Yield, 34%; Q(MCh), 151. The product was stored at 0° in the presence of a drop of $CHCl_3$. Adsorption of this material on acid-washed kieselguhr (British Drug Houses Ltd.), at pH 5·5–6 and elution with aqueous 10 mM ammonia effected no further purification.

RESULTS

Table 1 gives the rate of hydrolysis of the four substrates by the untreated brain homogenate and the partially purified fraction. It will be seen that purification has eliminated the hydrolysis of the aliphatic butyrate shown by the original homogenate. By contrast, the hydrolysis of *iso*amyl acetate was reduced relative to acetyl-β-methylcholine, but not eliminated. This shows that the hydrolysis of the butyrates was due to an enzyme, distinct from the cholinesterase, which contributes

* For definition of 'aceto-cholinesterase' see Sturge & Whittaker (1950).

to, but does not completely account for, the hydrolysis of *iso*amyl acetate. This conclusion is supported by experiments (lower part of Table 1) with 10 μM-eserine. This concentration of eserine inhibits cholinesterases but not ali-esterases (Richter & Croft, 1942). Acetyl-β-methylcholine hydrolysis is 100% inhibited in both preparations; the hydrolysis of the aliphatic butyrates is unaffected. The hydrolysis of *iso*amyl acetate by the purified

Table 1. *A comparison of unpurified and partially purified pigeon-brain cholinesterase*

	Untreated brain homogenate	Purified fraction
Q(MCh)	89	151
Rate of hydrolysis (% MCh rate)		
Tributyrin	18	<0·5
n-Amyl butyrate	4	1
*iso*Amyl acetate	71	53
Inhibition by 10 μM-eserine (%)		
Acetyl-β-methylcholine	100	100
*iso*Amyl acetate	76	100
Tributyrin	0	—
n-Amyl butyrate	0	—

Table 2. *Competition between aliphatic esters and acetyl-β-methylcholine for pigeon-brain cholinesterase*

Enzyme activity (μl./ml./hr.)			
Acetyl-β-methyl choline	*n*-Amyl acetate	*iso*-Amyl acetate	Mixed esters
832	264	—	764
1320	—	704	1160

fraction is abolished, but its hydrolysis by the original homogenate is not quite completely inhibited. The eserine-insensitive portion of the hydrolysis agrees well with the portion attributable to the ali-esterase in the unpurified homogenate. The results of Table 2 are consistent with competition of *n*- and *iso*-amyl acetate with acetyl-β-methylcholine for pigeon-brain cholinesterase. Although the purification procedure had effected but little concentration of cholinesterase activity, the primary object of the purification had thus been achieved in that the cholinesterase had been freed from ali-esterase activity. The specificity studies were therefore carried out with this preparation.

Specificity. As far as choline ester specificity is concerned, pigeon-brain cholinesterase behaves like a typical aceto-cholinesterase (Fig. 1; Fig. 2, upper portion). Acetylcholine shows the phenomenon of inhibition by excess substrate; the optimum substrate concentration is about 3 mM. Acetyl-β-methylcholine and propionylcholine are also split, though less rapidly, and butyrylcholine is hardly

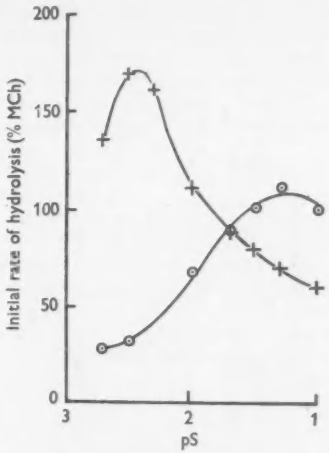

Fig. 1. Rate of hydrolysis of acetylcholine (×——×) and acetyl-β-methylcholine (⊙——⊙) as a function of substrate concentration. Ordinates: rate of hydrolysis as percentage of rate of hydrolysis of 30 mM-acetyl-β-methylcholine. Abscissae: negative logarithm of substrate concentration (pS).

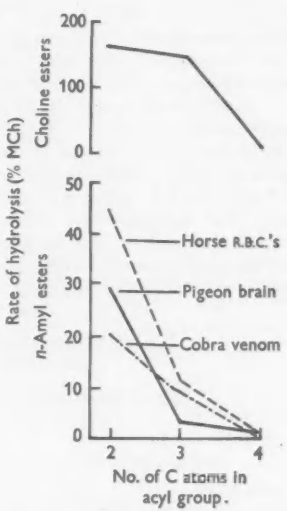

Fig. 2. Rate of hydrolysis of choline esters (upper portion) and *n*-amyl esters (lower portion) as a function of the size of the acyl group. Concentration of acetyl and propionylcholine, 3 mM; butyrylcholine, 10 mM.

attacked. Figs. 2 and 3 (continuous lines) show the relative rate of hydrolysis of a number of aliphatic esters by the pigeon-brain cholinesterase preparation. In Fig. 2 (lower portion) the rate of hydrolysis has been plotted against the number of carbon atoms in the acyl group of a series of *n*-amyl esters.

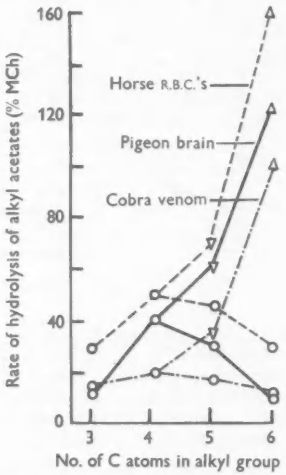

Fig. 3. Rate of hydrolysis of aliphatic acetates as a function of the size and configuration of the alkyl group. *n*-Alkyl acetates, ○; *iso*amyl acetate, △; 3:3-dimethylbutyl acetate, △.

As with the cholinesterases of horse erythrocytes (Mounter & Whittaker, 1950; broken lines) and cobra venom (Mounter, 1951; dotted and dashed lines) the rate of hydrolysis falls with increasing acyl-group size until with butyrate it is almost zero. A similar dependence on acyl-group size is seen with the choline esters (Fig. 2, upper portion). In Fig. 3 the effect of alkyl chain length and chain branching is presented. The abscissa now represents the number of carbon atoms in the alkyl group of a series of acetate esters; with the *n*-alkyl acetates the characteristic maximum at *n*-butyl acetate is once again observed as is the marked increase in rate on quaternization of the 3-carbon atom of *n*-butyl acetate. The specificity of pigeon-brain cholinesterase thus conforms precisely to the general pattern observed with the other aceto-cholinesterases.

Localization of avian-brain cholinesterase. During the purification of pigeon-brain cholinesterase, the impression was gained that the enzyme was attached to finely divided particulate material of varying particle size. The enzyme did not appear to be attached to grosser cell debris because, although much of it sedimented in centrifugal fields of low intensity (approx. 2000 *g*), washing these coarse particles or centrifugation of more dilute homogenates left relatively little activity in the precipitate. On the other hand, there was no evidence for two distinct 'fractions' of cholinesterase as described by Little (1948*a*, *b*) in mouse and dog brain, since higher speed centrifugation (16000 *g*) brought down appreciable proportions of the material in suspension at the lower speeds. It seemed likely that Little's 'supernatant' cholinesterase was merely a suspension of particles too fine to sediment in the ordinary laboratory centrifuge. To test this, dilute (2%) homogenates of mouse brain were prepared as described by Little (1948*a*) using water and 75 mM-sodium chloride. They were spun at 2000 *g* for 10 min. The distribution of activity in precipitate and supernatant was approximately as found by Little (Table 3). The supernatants were then spun at 16000 *g* for 30 min. There was some loss of activity in the water supernatant and considerable loss in the salt supernatant. Of the recovered activity, the greater part (72 and 77% respectively) was recovered in the precipitate. No doubt higher speeds and longer times would have led to even higher recoveries in this fraction. There is thus no evidence for separate forms of cholinesterase in brain, and the enzyme must be regarded as attached to particulate material as in the case of the red cell (Croft & Richter, 1943; Paléus, 1947; Augustinsson, 1948; Mounter & Whittaker, 1950). Probably salt concentration and other factors can

Table 3. *Distribution of mouse-brain cholinesterase between precipitate and supernatant of centrifuged homogenates*

(Figures in brackets are results of Little (1948*a*) inserted for comparison.)

Treatment	Percentage of recovered activity in: Precipitate	Supernatant	Activity lost (%)
2% Water homogenate:			
Homogenate centrifuged at 2000 *g*	5 (5)	95 (95)	0
Supernatant centrifuged at 16000 *g*	72	28	28
2% 75 mM-NaCl homogenate:			
Homogenate centrifuged at 2000 *g*	80 (65)	20 (45)	7
Supernatant centrifuged at 16000 *g*	77	23	—

influence the degree of aggregation of these particles as with the red cell preparations.

The localization of cholinesterase in mammalian brain has been the subject of several publications (Nachmansohn, 1939; Pighini, 1938; Birkhäuser, 1940; Augustinsson, 1948; Little, 1948*b*; Zeller, 1949; Burgen & Chipman, 1951; Ord & Thompson, 1952), but Nachmansohn's (1939) study of the domestic fowl appears to be the only systematic work with an avian brain. Nachmansohn found a considerably higher cholinesterase activity in the brain of this species than in mammalian brain, as the author has with the pigeon. Nachmansohn also showed that this high level of activity was a property of all parts of the brain, the optic lobes possessing a particularly high level of activity. This has been confirmed with the chick and is also true of the pigeon (Table 4). It is in marked contrast to mammalian brain, in which very varying levels of activity occur in different regions. Most authors have commented upon the high concentration of cholinesterase in the mammalian caudate nucleus and other basal ganglia, and the relatively low activity of the mammalian cortex. As the absolute activities given by different workers are somewhat difficult to compare, some figures for three mammalian species are included in Table 4 for comparison.

Table 4. *Cholinesterase activity of brains of various species*

(Determinations were carried out on pooled brains. Figures in brackets refer to the number of brains pooled.)

Avian brains		Mammalian brains	
Species	Q(MCh)	Species	Q(MCh)
Pigeon (4):		Cat (1)	22
Whole brain	87	Mouse (2)	30
Hemispheres	95	Rabbit (4):	
Optic lobes	147	Whole brain	26
Cerebellum	70	Caudate nucleus	105
Brain stem	43		
Chick* (6):			
Whole brain	88		
Hemispheres	86		
Optic lobes	143		
Cerebellum	72		
Brain stem	62		

* 5-day-old light Sussex cockerels.

DISCUSSION

The high level of avian-brain cholinesterase activity merits discussion. The phylogeny of the avian brain presents many difficulties, but it seems clear that the pallium of the primitive brain, from which the mammalian cortex is developed, is poorly represented in the avian telencephalon. The greater part of the forebrain represents a development of striatal tissue, from which the mammalian basal ganglia are also derived (cf. Kappers, Huber & Crosbie, 1936). The relatively higher cholinesterase content of the avian forebrain may thus be connected with the higher proportion of striatal tissue in this brain as compared with the mammalian brain. This does not, however, account for the high activity of the optic lobes, cerebellum and even the brain stem. No doubt the lower proportion of fibre tracts in the avian brain is an important factor (Nachmansohn, 1939). In the dog, the anterior lobe of the cerebellum, which may be more truly homologous with the avian cerebellum than the remaining portions of this organ, has a high activity, second only to the basal ganglia (Burgen & Chipman, 1951). The thalamus, corpora gemina and geniculate bodies, which in some degree must be regarded as homologous with the avian optic lobes, are also fairly active tissues.

The uneven distribution of the acetylcholine-aceto-cholinesterase-choline acetylase system in the various synaptic areas of the mammalian brain has led to the concept of two types of synaptic transmission in the central nervous system, cholinergic and non-cholinergic (Feldberg & Vogt, 1948). Possibly birds possess a greater proportion of the cholinergic synapses in their central nervous system, and a more detailed study of the localization of the components of the cholinergic system in avian brains might well contribute to the solution of this problem.

SUMMARY

1. Pigeon-brain cholinesterase has been shown to possess the specificity pattern characteristic of aceto-cholinesterases.

2. The high level of cholinesterase activity in the avian brain is confirmed and discussed.

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Gladiolic Acid, a Metabolic Product of *Penicillium gladioli*

2. STRUCTURE AND FUNGISTATIC ACTIVITY

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Gladiolic acid, an antifungal metabolic product of *Penicillium gladioli* Machacek has been shown to have the tautomeric structure I (Grove, 1952*a*; Raistrick & Ross, 1952). Ultraviolet and infrared spectroscopic investigation of gladiolic acid and its derivatives (Grove, 1952*b*) showed that whilst gladiolic acid was present in the lactol form I*a* (R = OH) in the solid state, an equilibrium between the lactol and open chain I*b* (R = OH) forms existed in aqueous solution, the pH being the determining factor. Furthermore, the ultraviolet-absorption spectrum of gladiolic acid in alkaline solution showed that the gladiolic acid anion had the hydrated (dihydroxyphthalan) structure II (R = O^-) although, as regards chemical activity, this structure was equivalent to I*b* (R = O^-); similarly, *o*-phthalaldehyde also existed in aqueous solution in the hydrated form (dihydroxyphthalan). In addition, the esters of gladiolic acid described by Grove (1952*a*) were shown to be pseudo esters of general formula I*a*.

Brian, Curtis & Hemming (1948) found that gladiolic acid inhibited germination of the spores of a number of fungi in Czapek-Dox medium at low pH, and Smith (1952) has presented data on the fungistatic activity of the related cyclopaldic and cyclopolic acids and on dihydrogladiolic acid. In the present paper the results of tests on a comprehensive series of derivatives of gladiolic acid and of simple model compounds chemically related to gladiolic acid are reported, and conclusions are drawn regarding the structural groupings which are responsible for the fungistatic properties of the gladiolic acid molecule. In addition, a number of other factors which influence the fungistatic activity of gladiolic acid are discussed in relation to the specificity shown by the antibiotic.

EXPERIMENTAL

Materials. The preparation and properties of the derivates of gladiolic acid are described by Grove (1952*a*). Model compounds were prepared by standard methods and purified by distillation or crystallization.

Routine evaluation of fungistatic activity (Tables 1 and 2). Compounds were assayed by the spore-germination test with conidia of *Botrytis allii* Munn. (Brian & Hemming, 1945; Brian *et al.* 1948), but Czapek-Dox was used in place of Weindling medium throughout, since gladiolic acid is unstable in the latter. All solutions were adjusted to pH 3·5 before assay, and dilutions made in $\times 2$ steps. Small discrepancies (within the limits of error arising from such a dilution procedure) between the figures quoted for the fungistatic concentrations of certain compounds in different sections of this paper are due to the dilution of solutions of differing initial concentration. Salicylanilide was used throughout as standard reference substance; all tests were carried out in duplicate and the mean figures quoted in the tables. The highest concentration tested was limited by the water solubility of the particular compound under investigation.

Evaluation of fungistatic activity at different pH *values* (Tables 4 and 5). This was made in the same way as in the routine assay at pH 3·5 (above) by direct comparison with controls at the appropriate pH. Thus the figures quoted for 98–100% inhibition of germination are independent of the variation of germination with pH, which is, in any case, almost negligible for *B. allii* between pH 3 and 8.

Stability of lactol and aldehyde acetates in aqueous solution

In buffer solution. The compounds (50 mg.) in ethanol (2 ml.) were added to McIlvaine buffer (10 ml.), and the solutions allowed to stand at 25° (in some cases the starting material slowly separated) with occasional shaking. After 3 days the acid liberated by hydrolysis was estimated by titration with 0·1 N-NaOH to the first appearance of a pink colour with phenolphthalein (simultaneous titration of a blank).

Compound	Hydrolysis (%) at pH 3·9	Hydrolysis (%) at pH 7·2
Tetraacetyl-*o*-phthalaldehyde dihydrate	0	2·6
Triacetylgladiolic acid hydrate	6·4	5·4
Methyl gladiolate	4·0	6·0

In alkaline medium. The neutral compounds (50 mg.) in 30% (v/v) aqueous ethanol (10 ml.) were titrated with 0·1 N-NaOH to the phenolphthalein end point in CO_2-free air. The red colour produced after the addition of the first drops of alkali rapidly disappeared, in some cases within a few seconds and the titration was continued in this manner, adding alkali two drops at a time, until a pink colour was obtained which remained permanent for 3 hr.

The compounds varied considerably in their behaviour towards hydrolysis under these conditions. Thus, diacetylphenylglyoxal hydrate titrated almost like a dibasic acid and hydrolysis was complete within a few minutes. Acetylgladiolic acid (potential dibasic acid), 3-acetoxyphthalide (dibasic), triacetylgladiolic acid hydrate (tetrabasic) and tetraacetyl-*o*-phthalaldehyde dihydrate (tetrabasic) rapidly consumed alkali at first, but after a time the rate of hydrolysis slowed (titration time to the arbitrary end point (above), 2 hr.; % hydrolysed, 80–90), and the behaviour was more akin to that of a readily opened lactone. Methyl gladiolate was more stable and slowly consumed 0·9 equivalent in 3 hr.

Ultraviolet-absorption spectra of gladiolic acid (Fig. 1). Appropriate quantities of gladiolic acid were dissolved by shaking at room temperature in McIlvaine or Clark and Lubs buffer solutions, the pH values checked on a Cambridge pH meter, and the ultraviolet spectra obtained with a Unicam S.P. 500 spectrophotometer.

It has been shown (Grove, 1952*b*), by comparison with model compounds of known structure, that the ultraviolet-absorption curve *A* (Fig. 1) with maxima at 2670 and 3050 A is given by the lactol form (I*a*, R = OH) of gladiolic acid and is obtained in aqueous solutions of low pH, whereas curve *B* is characteristic of the hydrated open chain form II and is obtained at high pH values. When the ultraviolet absorption of gladiolic acid is examined at pH values between these extremes, a family of curves of intermediate shape is obtained and from the value

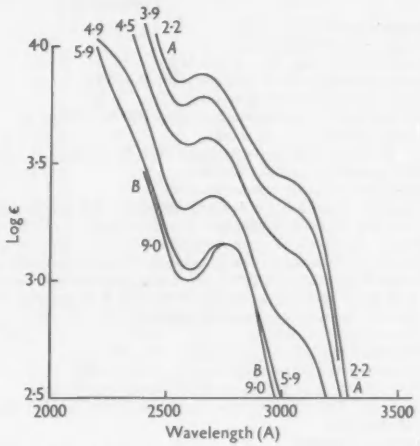


Fig. 1. Ultraviolet absorption spectra of gladiolic acid at different pH values.

of the extinction coefficient at 2670 A it is possible to calculate the percentage of lactol form present. The percentages of open-chain form obtained by subtraction from 100 are compared in Table 3 with the percentages of dissociated molecules calculated from the pK (4·4).

RESULTS

The concentration of gladiolic acid and a number of its derivatives required to produce 98–100% inhibition of germination of the spores of *B. allii* are given in Table 1 and compared with salicylanilide and mercuric chloride. Similar data for the model compounds related to gladiolic acid are collected in Table 2.

The stabilities of acetylgladiolic acid and gladiolic acid at different pH values are compared in Table 4.

Table 5 compares the effect of pH on the fungistatic activity of gladiolic acid and the neutral derivatives acetylgladiolic acid and triacetylgladiolic acid hydrate, with three neutral compounds tetraiodoethylene, *o*-phthalaldehyde and 3-acetoxyphthalide. The logarithms of the equieffective concentrations of these compounds are plotted against pH in Fig. 2.

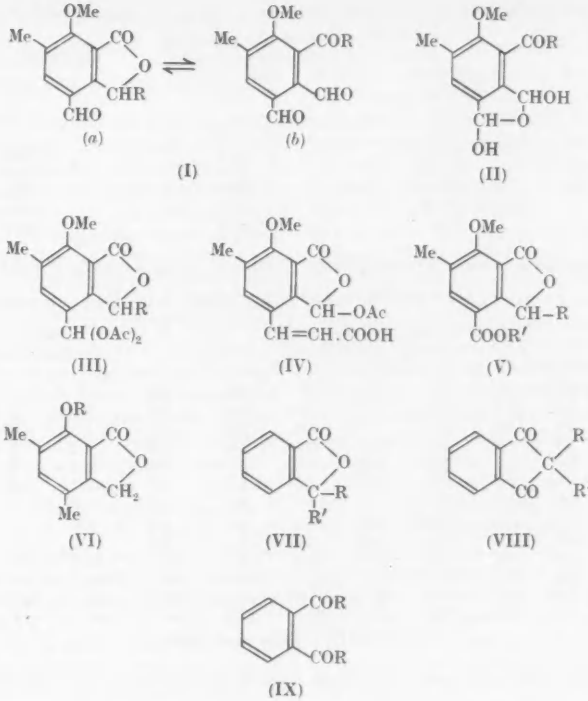
The results of tests with some of these compounds as inhibitors of spore germination for a wide range of fungi are given in Table 6.

Table 1. *Fungistatic activity of gladiolic acid derivatives*

Compound	Concentration inhibiting germination of *Botrytis allii* spores at pH 3·5 (μg./ml.)	($M \times 10^{-5}$)
Gladiolic acid (I, R = OH)	6·25	2·8
Gladiolic acid hemihydrate	6·25	2·7
Acetylgladiolic acid (I*a*, R = OAc)	1·6	0·6
Triacetylgladiolic acid hydrate (III, R = OAc)	6·25	1·7
Methyl gladiolate (I*a*, R = OMe)	12·5	5·3
Diacetyl methyl gladiolate hydrate (III, R = OMe)	25·0	7·4
Ethyl gladiolate (I*a*, R = OEt)	12·5	5·0
Diacetyl ethyl gladiolate hydrate (III, R = OEt)	6·25	1·8
Ethyl gladiolate semicarbazone	>100	>32·3
Acetylgladiolic acid semicarbazone	62·5	19·5
3-Acetoxy-7-methoxy-6-methylphthalidyl-4-β-acrylic acid (IV)	>100	>32·7
7-Methoxy-6-methylphthalide-4-carboxylic acid (V, R = R′ = H)	>100	>45·0
Methyl 7-methoxy-6-methylphthalide-4-carboxylate (V, R′ = Me, R = H)	>100	>42·4
3-Hydroxy-7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = OH, R′ = H)	>100	>42·0
Methyl 3-hydroxy-7-methoxy-6-methylphthalide-4-carboxylate (V, R = OH, R′ = Me)	>100	>39·7
3-Acetoxy-7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = OAc, R′ = H)	>100	>35·7
4-Formyl-7-methoxy-6-methylphthalide (I*a*, R = H)	>100	>48·5
Dihydrogladiolic acid	>100	>44·7
7-Methoxy-4:6-dimethylphthalide (VI, R = Me)	>100	>52·1
7-Hydroxy-4:6-dimethylphthalide (VI, R = H)	>100	>56·2
4-Methoxy-5-methylbenzene-1:2:3-tricarboxylic acid	>100	>39·4
4-Methoxy-5-methylbenzene-1:2:3-tricarboxylic acid trimethyl ester	>100	>33·8
Salicylanilide	62·5	29·3
Mercuric chloride	0·1	0·04

Table 2. *Fungistatic activity of some compounds related to gladiolic acid*

Compound	Concentration inhibiting germination of *Botrytis allii* spores at pH 3·5 (μg./ml.)	($M \times 10^{-5}$)
Benzoic acid	125	102
Methyl benzoate	>1000	>735
Benzaldehyde	100	94·3
Phenyl glyoxal	125	93·3
Diacetylphenyl glyoxal hydrate	>100	>42·4
Dimethyl phthalate	>100	>51·5
Phthalaldehydic acid (VII, R = H, R′ = OH)	>100	>66·7
Phthalide (VII, R = R′ = H)	>100	>74·6
3-Methoxyphthalide (VII, R = H, R′ = OMe)	>100	>61·0
3-Acetoxyphthalide (VII, R = H, R′ = OAc)	50	26·0
o-Acetylbenzoic acid (VII, R = Me, R′ = OH)	>100	>61·0
3-Methoxy-3-methylphthalide (VII, R = Me, R′ = OMe)	>100	>56·2
Benzil-2-carboxylic acid (VII, R = COPh, R′ = OH)	>100	>39·4
3-Acetoxy-3-benzoylphthalide (VII, R = COPh, R′ = OAc)	>100	>33·8
Phthalyl alcohol	>100	>72·5
o-Formylbenzyl alcohol	>100	>73·5
1:3-Diketoindane (VIII, R = R′ = H)	100	68·5
Triketohydrindene hydrate (VIII, R = R′ = OH)	>500	>181
2:2-Diacetoxy-1:3-diketoindane (VIII, R = R′ = OAc)	>100	>38·2
1:2-Diacetylbenzene (IX, R = Me)	>100	>61·8
Terephthalaldehyde	500	373
*iso*Phthalaldehyde	500	373
Tetraacetyl-*o*-phthalaldehyde dihydrate	100	28·8
o-Phthalaldehyde (IX, R = H)	6·5	4·8
Gladiolic acid (4-methoxy-5-methyl-*o*-phthalaldehyde-3-carboxylic acid)	6·25	2·8
Salicylanilide	62·5	29·3



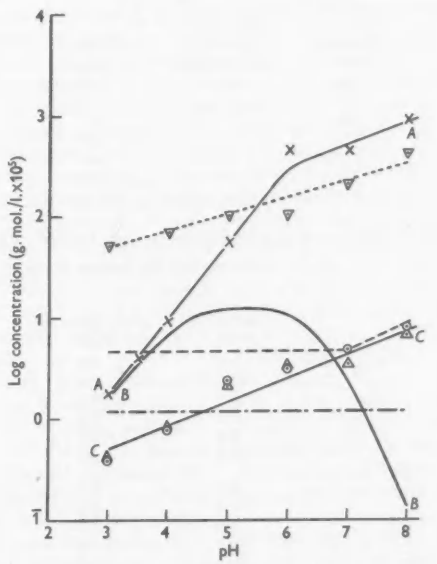


Fig. 2. Equieffective concentrations of fungistatic compounds at different pH values. ▽, 3-Acetoxyphthalide; – – –, *o*-phthalaldehyde; —.—, tetraiodoethylene; ×, *A*, gladiolic acid; *B*, gladiolic acid (undissociated molecules); ⊙, *C*, acetylgladiolic acid; △, triacetylgladiolic acid hydrate.

Table 3. *Effect of* pH *on the tautomeric equilibrium I and on the dissociation of gladiolic acid*

pH	ϵ_{2670} (data from Fig. 1)	Hydrated open-chain (dihydroxyphthalan) form (II) (%)	Dissociated molecules (calc. from pK) (%)
2·2	7600	0	0·6
3·0	7500	1·6	3·8
3·9	6030	24·0	24·0
4·5	3980	55·3	55·7
4·9	2190	82·6	76·0
5·9	1170	98·2	97·0
9·0	1050	100·0	100·0

Table 4. *Fungistatic activity of acetylgladiolic acid and gladiolic acid in Czapek-Dox medium*

		Concentrations inhibiting germination of *Botrytis allii* spores (M × 10^{-5})									
		Acetylgladiolic acid Storage at 25° (days)					Gladiolic acid* Storage at 25° (days)				
	pH of solution	0	1	2	3	16	0	1	2	3	16
(*a*) Assayed at pH 3·5	3·0	0·4	0·4	0·4	0·4	0·8	3·6	4·7	4·7	3·6	4·7
	4·0	0·4	0·4	0·4	0·4	2·4	3·6	4·7	7·2	4·7	3·6
	5·0	0·4	0·4	0·8	0·4	2·4	3·6	7·2	4·7	7·2	4·7
	6·0	0·4	0·8	2·4	0·8	4·7	3·6	4·7	4·7	4·7	4·7
	7·0	0·4	0·8	4·7	2·4	4·7	3·6	7·2	4·7	3·6	4·7
	8·0	0·4	2·4	4·7	4·7	4·7	3·6	7·2	7·2	4·7	4·7
(*b*) Assayed at pH of solution	3·0	0·4	0·4	0·4	0·4	1·2	1·8	3·6	1·8	1·8	1·8
	4·0	0·8	2·4	2·4	2·4	1·2	9·4	9·4	7·2	7·2	9·4
	5·0	2·4	3·2	4·7	9·5	2·4	56·3	56·3	56·3	56·3	56·3
	6·0	3·2	18·9	18·9	37·9	18·9	450	450	450	450	450
	7·0	4·7	37·9	37·9	37·9	37·9	450	450	450	450	450
	8·0	9·5	37·9	37·9	37·9	37·9	900	900	900	450	900

* Calculated from Brian *et al.* (1948).

Table 5. *Fungistatic activity of compounds assayed in Czapek-Dox medium at varying* pH

	Concentration (M × 10^{-5}) causing inhibition of germination of *Botrytis allii* spores					
pH of solution	Gladiolic acid*	Acetyl-gladiolic acid	Triacetyl-gladiolic acid hydrate	*o*-Phthal-aldehyde	Tetraiodo-ethylene	3-Acetoxy-phthalide
3·0	1·8	0·4	0·4	4·7	1·2	52·1
4·0	9·4	0·8	0·85	4·7	1·2	69·5
5·0	56·3	2·4	2·3	4·7	1·2	104
6·0	450	3·2	3·4	4·7	1·2	104
7·0	450	4·7	3·4	4·7	1·2	208
8·0	900	9·5	6·8	9·4	1·2	416

* Calculated from Brian *et al.* (1948).

Table 6. *Fungistatic activity of gladiolic acid, acetylgladiolic acid, methyl gladiolate and* o*-phthalaldehyde*

	Concentrations causing inhibition of germination at pH 3·5 (M × 10^{-5})			
	Gladiolic* acid	Acetylgladiolic acid	Methyl gladiolate	*o*-Phthalaldehyde
Absidia glauca Hagem	0·9	0·6	10·6	0·1
Aspergillus niger van Tiegh.	56·3	2·4	42·4	1·2
Botrytis allii Munn.	3·5	0·6	21·2	4·7
Fusarium coeruleum (Lib.) Sacc.	1·8	0·3	10·6	0·1
F. graminearum Schwabe	0·4	0·1	21·2	0·6
Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr.	28·2	9·5	42·4	9·4
Mucor erectus Bain	3·5	0·3	21·2	0·6
Penicillium digitatum Sacc.	7·0	2·4	21·2	1·2
P. expansum Link	1·8	1·2	21·2	0·1
P. gladioli Machacek	3·5	2·4	>42·4	0·3
Stemphylium sp.	28·2	4·8	21·2	2·4
Thamnidium elegans Link	28·2	0·1	42·4	4·8
Trichoderma viride Pers. ex Fr.	113·0	38·0	>42·4	37·3

* Calculated from Brian *et al.* (1948).

DISCUSSION

Effect of structure on fungistatic activity

Most simple aromatic compounds with functional substituents are fungistatic at concentrations of the order of 10^{-3}–10^{-2} M, provided that the pH of the assay medium is so adjusted that ionization of acidic or basic substances is reduced to a minimum. The commercially important fungicidal compound salicylanilide inhibits germination of *B. allii* spores at $2{\cdot}9 \times 10^{-4}$ M at pH 3·5, but at this pH gladiolic acid is fungistatic at much lower concentrations (of the order of 10^{-5} M). For the purposes of the present paper, therefore, the term 'active' is applied to those compounds showing fungistasis in the same concentration range as gladiolic acid; compounds causing no inhibition of germination at concentrations greater than the lowest fungistatic concentration of salicylanilide are designated 'inactive'. It must, nevertheless, be borne in mind that gladiolic acid is only weekly fungistatic compared with mercuric chloride (Table 1). The maximum fungistatic activity of gladiolic acid (pK 4·4) is not observed in the routine assay (see later), nevertheless, it has been found more convenient to carry out the assay at this pH.

Examination of the results obtained with the model compounds (Table 2) shows that benzoic acid, phenylglyoxal and benzaldehyde are all fungistatic at a concentration of approx. 10^{-3} M, but *o*-phthalaldehyde (IX, R = H) is active at 5×10^{-5} M, i.e. in the same concentration range as gladiolic acid. Phthalyl alcohol, *o*-formylbenzyl alcohol, phthalaldehydic acid and dimethyl phthalate, all compounds closely related to *o*-phthalaldehyde but at a different level of oxidation or reduction, are inactive, as are phthalide and all the 3-substituted phthalides tested. The importance of the two formyl substituents is brought out by the fact that not only are compounds of structure (VIII), including 1:3-diketoindane and triketohydrindene hydrate and its diacetyl derivative, inactive, but so also is 1:2-diacetylbenzene (IX, R = Me). That the two formyl substituents must be in the ortho position on the aromatic nucleus is emphasized by the inactivity of *iso*- and *tere*-phthalaldehydes.

The only active compounds in the series of gladiolic acid derivatives (Table 1), namely gladiolic acid, acetylgladiolic acid (I*a*, R = Ac), triacetylgladiolic acid hydrate (III, R = OAc), the pseudo esters (I*a*, R = OMe and OEt) and the acetyl derivatives of their hydrates, are those in which both formyl groups are either intact or readily available on hydrolysis. The semicarbazones of acetylgladiolic acid and ethyl gladiolate show weak activity of the same order as salicylanilide. But all derivatives in which one or both of the formyl substituents has been oxidized to —COOH (e.g. 3-hydroxy-7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = OH, R′ = H), 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid and their neutral esters) or reduced to $—CH_2OH$ (dihydrogladiolic acid, 4-formyl-7-methoxy-6-methylphthalide (I*a*, R = H) or $—CH_3$ (7-methoxy-4:6-dimethylphthalide (VI, R = Me)) are inactive; *iso*gladiolic acid (7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = R′ = H)), and its neutral methyl ester are also inactive. Thus, of the known metabolic products of *P. gladioli*, namely, gladiolic acid, dihydrogladiolic acid, 4-formyl-7-methoxy-6-methylphthalide and 7-hydroxy-4:6-dimethylphthalide (VI, R = H), only gladiolic acid shows fungistatic activity.

The inactivity of 4-formyl-7-methoxy-6-methylphthalide (I*a*, R = H), taken in conjunction with the results on the model compounds already discussed, suggests that the lactol form (I*a*, R = OH) of the tautomeric system (I) is inactive *per se*. It can be argued that the activity shown by acetylgladiolic acid (I*a*, R = OAc) and the pseudo esters (I*a*, R = OMe and OEt) does not support the conclusion, but this objection is dealt with below. Like gladiolic acid, *o*-phthalaldehyde is rapidly inactivated in Weindling medium at pH 5 or above with the production of dark-green or greenish brown solutions. Moreover, the morphological effects produced by marginal concentrations of *o*-phthalaldehyde on the germ tubes of *B. allii* are identical with those produced by similar concentrations of gladiolic acid. One must conclude, therefore, that gladiolic acid owes its fungistatic activity to the *o*-phthalaldehyde grouping present in the open chain form (I*b*, R = OH), and that this grouping, which undergoes hydration in aqueous solution, probably reacts with some specific cell constituent.

Good agreement (within the limits of experimental error) is obtained when the percentage of dissociated molecules is compared with the percentage of open chain form present at a given pH (Table 3). It follows that in any investigation of the effect of pH on the fungistatic activity of gladiolic acid, the undissociated lactol form (I*a*, R = OH) and the hydrated open chain anion (II, R = O^-) are the most important molecular species to be considered. It is therefore difficult to escape the conclusion that the anion is the principal active form of gladiolic acid. The undissociated open-chain forms (I*b*, R = OH or II, R = OH) in equilibrium with (I*a*, R = OH) and (II, R = O^-) may be present, undetected by the spectroscopic technique employed, but together probably not exceeding 1 % of the total concentration of gladiolic acid, and may contribute to the total activity; but if (I*b*, R = OH) or (II, R = OH) were the sole active species, the effective external fungistatic concentration of active species

would be of the order of 10^{-7} M or less, an entirely different order of concentration from that found with *o*-phthalaldehyde.

Effect of ionization on fungistatic activity

Brian *et al.* (1948) concluded that the effect of the pH of the external medium on the fungistatic activity of gladiolic acid was best explained on the assumption that only undissociated molecules of gladiolic acid penetrated the plasma membrane of the spore. As is shown below, this is not now thought to be wholly correct; nevertheless, the undissociated molecule is the most effective penetrating species. Thus the molecular species which can most readily penetrate the plasma membrane is inactive, while the species which is mainly responsible for fungistatic activity, the hydrated open-chain anion, has greater difficulty in effecting penetration.

Two factors, therefore, penetration and the internal availability at the site of action of the active molecular species, operate in determining the fungistatic power of gladiolic acid, and these factors are determined respectively by the external and internal pH values. Thus, a low external pH favours penetration and a high internal pH favours dissociation to give the active anion.

Unfortunately, nothing is known about the internal pH of *B. allii* spores, and how this might be expected to vary with the external pH. Nevertheless, it is possible to make some predictions of a general nature by analogy with published work on the mycelium of other organisms. Thus if we assume that the (largely aqueous) cytoplasm of the *B. allii* spores is separated from the external medium by a chitinous cell-wall and lipoprotein semipermeable 'membrane', then in Czapek-Dox medium the internal and external concentration of ions will be governed by a Donnan equilibrium. Robbins (1924) found the isoelectric points of pH 5·0 and 5·5 for the intracellular colloids of *Rhizopus nigricans* and *Fusarium lycopersici* respectively. Most workers have found the internal pH of mycelium to fall between 4·8 and 6·5 (Armstrong, 1929; Mahdihassan, 1930), and there is evidence that the internal pH is well buffered against changes in the external pH. Thus Bünning (1936) found the internal pH of mycelium of *A. niger* only altered from 4·4 to 7·0, while the external pH was changed from 2·5 to > 8·0. The most likely internal pH for the *B. allii* spore would seem therefore to be about 5, being but little affected by changes in external pH of the order studied in the present investigation. Nevertheless, the pH may vary in different parts of the cytoplasm and it is the pH at the site of action which is critical.

Since the pH at which the assay is carried out has such a profound influence on the fungistatic activity of gladiolic acid (Brian *et al.* 1948), it is instructive to examine the effect of varying the pH of assay on the activity of some of the derivatives of gladiolic acid and on *o*-phthalaldehyde (Tables 4 and 5). In the series assayed at pH 3·5 (Table 4*a*), acetylgladiolic acid appears to be less stable than gladiolic acid; however, the concentration of acetylgladiolic acid effective after standing 16 days at pH 8·0 is close to the concentration of gladiolic acid normally effective at the pH of assay (3·5), suggesting that the fall in activity is due to slow hydrolysis of acetylgladiolic acid to gladiolic acid in the external medium. In the series assayed at different pH values (Table 4*b*), acetylgladiolic acid is much less affected by the pH of assay than is gladiolic acid. The concentration of tetraiodoethylene, a stable neutral fungistatic compound (Muirhead, 1949), effective against *B. allii* spores, is independent of the pH of assay (Table 5). *o*-Phthalaldehyde behaves similarly, the slight fall in activity at pH 8 being due to decomposition of the material which occurs readily in the presence of hydroxyl ions (Grove, 1952*b*). It follows either that penetration of a non-electrolyte, and in particular a neutral dialdehyde, to the site of action and the equieffective concentrations at that site are independent of the pH of the external medium, or that any variations in the ability to penetrate at different pH values are exactly balanced by variations with pH of the equieffective concentration at the site of action. The former is more probable and is used in subsequent arguments.

The logarithms of the equieffective concentrations of gladiolic acid plotted against pH (Fig. 2, curve *A*) fall on a line which is initially linear and steeply inclined to the pH axis but flattens out above pH 6. The corresponding equieffective concentrations of undissociated molecules (curve *B*) derived from these values fall away rapidly at pH values greater than 6 and the general picture is typical of that shown by weak acids (Beevers & Simon, 1949; Simon, 1950). Simon has pointed out that since a constant external concentration of undissociated molecules does not produce the same response at each pH level, it cannot he held that the undissociated molecules are solely responsible for toxicity. Indeed, in the case of gladiolic acid, it seems probable from the chemical evidence that toxicity is due to the ion and this may therefore be true for other weak acids which give similar curves. (For summary, see Simon & Blackman, 1949.)

Acetylgladiolic acid and triacetylgladiolic acid hydrate (Fig. 2) give a linear plot of the logarithms of the equieffective concentrations against pH unlike that of the weak acid gladiolic acid or the non-electrolyte tetraiodoethylene. It will be recalled (Table 1) that while acetylgladiolic acid and triacetylgladiolic acid hydrate are more active than gladiolic acid at pH 3·5 (partly due to the absence

of the ionization effect mentioned above), the pseudo esters (I*a*, R = OMe and OEt) and the acetyl derivatives of their hydrates are appreciably less active. These results, and those in Table 4, might be explained on the hypothesis that these neutral gladiolic acid derivatives penetrate the plasma membrane of the spore unchanged but are hydrolysed (by enzyme action) within the cell to gladiolic acid which is the active principle. It is suggested, moreover, that the differences in activity between these compounds are due to their differing susceptibilities to enzymic hydrolysis, paralleled by their behaviour towards acid hydrolysis *in vitro*. Lactol acetates, aldehyde acetates and the pseudo esters of gladiolic acid are all rapidly hydrolysed in alkaline solution at room temperature but are relatively stable in acid solution, tetraacetyl-*o*-phthalaldehyde dihydrate being the most and triacetylgladiolic acid hydrate the least stable. The inactivity of tetraacetyl-*o*-phthalaldehyde dihydrate can thus be explained on the above hypotheses. The logarithms of the equieffective concentrations of 3-acetoxyphthalide plotted against pH lie on a line of similar slope to the acetyl derivative of gladiolic acid (though at 100 times the concentration), and it is clear that this is an effect characteristic of this type of compound which is readily hydrolysed to a weak electrolyte.

It has been shown that the susceptibility of triacetylgladiolic acid hydrate towards hydrolysis does not alter significantly between pH 3·9 and 7·2. One may conclude that it is unlikely that the increase in the equieffective concentration of this substance between these pH limits is due to increasing hydrolysis in the external medium.

A simplified diagram of the processes involved in the penetration of the acetyl derivatives of gladiolic acid is given in Fig. 3; internal hydrolysis to gladiolic acid is assumed to go almost to completion. If the penetration is a passive process then the equieffective external concentrations [AcA_e] (Fig. 2, curve *C*) will be proportional to the equieffective total internal concentrations of gladiolic acid ([HA_i]+[A_i^-]) resulting from the external application of these substances. In Table 7 the variation of internal with external pH found by Bünning (1936) has been assumed to hold for the *B. allii* spores and internal concentrations corresponding to the equieffective external concentrations calculated in order to see whether a constant internal concentration of anion is obtained (cf. *o*-phthalaldehyde, above). This constant concentration of anion is not found, and indeed is only obtained in the unlikely event of the internal pH being low (approx. 3) when the external pH lies between 8 and 5 and then rises (to 4·5) as the external pH falls from 5 to 3. There is, of course, no valid reason for supposing that what appeared to hold true for a non-electrolyte would also hold true for the anion, but since small variations in internal pH are involved, a constant internal concentration of anion might be expected. K_1 is probably close to unity and independent of pH, but K_2 would be expected to be pH dependent; in addition, the pH at the (unknown) site of action may be very different from the bulk internal pH, and these factors may contribute to the observed deviations from expectation.

However, rather better agreement with expectation is obtained in the case of gladiolic acid. The

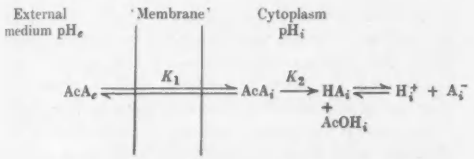


Fig. 3. Penetration of acetylgladiolic acid.

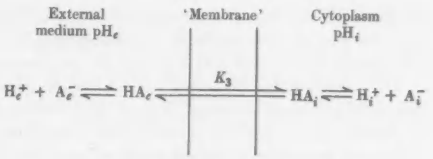


Fig. 4. Penetration of gladiolic acid.

AcA represents the molecule of acetylgladiolic acid, HA the undissociated molecule of gladiolic acid and A^- the gladiolic acid anion. The subscripts *e* and *i* denote external and internal, respectively. K_1, K_2 and K_3 are constants.

Table 7. *Equieffective internal concentrations of gladiolic acid from data on the acetyl derivatives* ($M \times 10^{-5}$)

External pH (pH_e)	Internal pH (pH_i) (assumed, after Bünning, 1936)	$K_1[HA_i]$	$K_1[A_i^-]$	$K_1([HA_i]+[A_i^-])$ (from Fig. 2, curve *C*)
3·0	4·5	0·2	0·2	0·4
4·0	4·5	0·4	0·4	0·8
5·0	4·5	0·7	0·9	1·6
6·0	5·0	0·5	2·0	2·5
7·0	5·5	0·3	3·7	4·0
8·0	7·0	0·03	8·97	9·0

Table 8. *Equieffective internal concentrations of gladiolic acid* (M × 10^{-5})

External pH (pH_e)	Internal pH (pH_i) (assumed, after Bünning, 1936)	$K_3[HA_i]$ (from Fig. 2, curve *B*)	$K_3[A_i^-]$	$K_3([HA_i]+[A_i^-])$
3·0	4·5	3·8	4·8	8·6
4·0	4·5	9·2	11·6	20·8
5·0	4·5	12·5	15·7	28·2
6·0	5·0	10·0	30·0	40·0
7·0	5·5	2·3	28·8	31·1
8·0	7·0	0·14	55·9	56·0

equilibria involved are represented diagrammatically in Fig. 4. The internal concentrations of undissociated molecules ($[HA_i]$) will be proportional to the corresponding external concentration ($[HA_e]$) but local potential barriers due to local differences in charge on the protein layers of the 'membrane', combined with the differing internal and external pH values would be expected to produce a large concentration gradient across the membrane of the undissociated molecules of a weak electrolyte.

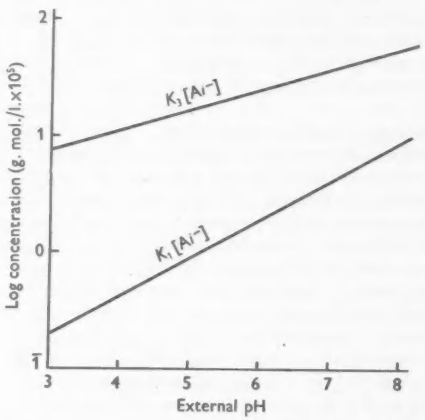


Fig. 5. Internal anion concentrations corresponding to the equieffective external concentrations of gladiolic acid and acetylgladiolic acid.

In Table 8 values of $K_3[A_i^-]$ corresponding to values of $K_3[HA_i]$ taken from Fig. 2, curve *B*, have been calculated for the same variation of internal with external pH as was assumed in Table 7. The logarithms of these values are plotted in Fig. 5 (together with the corresponding data for $K_1[A_i^-]$ from Table 7) and lie on a line only slightly inclined to the pH axis. The decrease in equieffective concentration below pH 4 (which is also apparent in Fig. 2, curve B) probably reflects the increasing contribution due to the penetration of gladiolic acid anions on the acid side of the isoelectric point.

To sum up. The effects of pH on the fungistatic activity of gladiolic acid can be broadly explained by the theory that the anion is the active molecular species but only the undissociated molecules penetrate the plasma membrane of the spore, and assuming a variation of internal and external pH that seems probable from the results of earlier work. Agreement is not perfect but is somewhat better for gladiolic acid than for acetylgladiolic acid which is assumed to penetrate unchanged but is then hydrolysed internally to gladiolic acid.

A great deal of the foregoing is of necessity speculative owing to the deficiencies in our knowledge of the system being studied, and the final elucidation of these problems must await further work, particularly on the biological side.

Specificity of fungistatic activity

Brian *et al.* (1948) tested gladiolic acid for inhibition of spore germination against a number of fungi and described a wide range of susceptibility among the fungi tested. The fungistatic concentrations at pH 3·5 of the neutral derivatives of gladiolic acid, acetylgladiolic acid and methyl gladiolate, and *o*-phthalaldehyde, fall into much narrower concentration bands when the substances are tested against the same fungi (Table 6), and although the variations from species to species are still quite large, the deviations from the means are significantly smaller than for gladiolic acid.

The fungistatic concentration of *o*-phthalaldehyde is of the order of 10^{-5} M for all the fungi tested, and is independent of the external pH between 3 and 7; gladiolic acid shows more marked specificity, and fungistatic concentrations are highly dependent on external pH. It is reasonable to assume slightly different membrane potentials and internal pH values for each species of fungus at a given external pH; and near the pK of gladiolic acid, small changes in pH produce relatively large changes in the ratio of undissociated molecules to ions. It is difficult to escape the conclusion, therefore, that the enhanced specificity of action of gladiolic acid arises from the presence of the carboxyl substituent, which not only gives the molecule the penetration specificity characteristic of any weak acid, but also, being situated next to one of the formyl groups and taking part in the tautomeric system (I), limits the proportion of fungistatic molecules and makes it pH dependent.

SUMMARY

1. Gladiolic acid (4-methoxy-5-methyl-*o*-phthalaldehyde-3-carboxylic acid in the open-chain form) is the only fungistatic compound among the known metabolic products of *Penicillium gladioli* Machacek.

2. *o*-Phthalaldehyde shows fungistatic activity in the same concentration range as gladiolic acid and tests on a large number of derivatives of gladiolic acid and on simple model substances indicate that activity is connected with the presence of two formyl substituents in the ortho position on the aromatic nucleus. The lactol form of gladiolic acid (4-formyl-3-hydroxy-7-methoxy-6-methylphthalide) is believed to be inactive.

3. The percentage of dissociated molecules of gladiolic acid present at any given pH is in good agreement with the percentage of hydrated open-chain (dihydroxyphthalan) form calculated from the ultraviolet absorption. The undissociated lactol and hydrated open-chain anion make up 99% of the total gladiolic acid present at any given pH.

4. An attempt to explain the variation of the equieffective concentration of gladiolic acid with the external pH, on the basis that whereas the hydrated open-chain anion is the active species, only the undissociated lactol form penetrates into the spore, is not unsuccessful.

5. The variation with pH of the activity of acetylgladiolic acid and certain closely related neutral derivatives is not quite so satisfactorily explained on the hypothesis that the molecules penetrate unchanged but undergo internal enzymic hydrolysis to gladiolic acid.

6. The enhanced specificity of the fungistatic action of gladiolic acid compared with that of *o*-phthalaldehyde arises from the presence of the carboxyl substituent adjacent to the *o*-diformyl grouping.

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New Zealand Fish Oils

6. SEASONAL VARIATIONS IN THE COMPOSITION OF NEW ZEALAND GROPER (*POLYPRION OXYGENEIOS*) LIVER OIL

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The groper (*Polyprion oxygeneios*) is one of the larger and more important New Zealand edible fish. The fatty-acid composition of the head oil and of the liver oil has been described by Shorland (1938), while the vitamin A and vitamin D contents of the liver oils have been determined respectively by Shorland (1937, 1948, 1950), Cunningham (1935), Cunningham & Scott (1944) and Weeber (1945).

This paper describes the seasonal changes in size of liver, yield of oil, vitamin A, cholesterol, total unsaponifiable content, phospholipid and iodine value.

EXPERIMENTAL

Thirty-nine samples, each containing from 8 to 53 (average 24) livers, representative of a day's catch from one boat, were collected in the period 1938–43, at approximately equal intervals of time to minimize the possible differences between years. During the months of September and October, however, when there was invariably a marked increase in vitamin A potency, eleven samples were taken as

compared with five or six for the remaining 2-monthly periods. The fish were all from Cook Strait and the lipids were extracted as described by Oliver & Shorland (1948).

Vitamin A in the samples was determined from the $E^{1\%}_{1\,cm.}$ values in absolute ethanol at 328 mμ. using the conversion factor 1/16 for expressing it as a percentage vitamin A (cf. Carr & Jewell, 1933). No correction was made for irrelevant absorption but close agreement was obtained with the percentage vitamin A determined from the $SbCl_3$ blue colour, assuming that the pure vitamin has an $E^{1\%}_{1\,cm.}$ 620 mμ. value of 4800 (Baxter & Robeson, 1942).

In recent years (cf. Cama, Collins & Morton, 1951), higher values for the $E^{1\%}_{1\,cm.}$ absorption of vitamin A have been found using purer samples of this substance and the more exacting methods of analysis now available (Morton & Stubbs, 1946, 1948) require more absorption data than was obtained at the time of analysis. In any case the possible error involved is insufficient to affect the interpretation of the present results.

Iodine values were determined by the Wijs method, the percentage unsaponifiable matter by the Society of Public Analysts' Procedure (1933), and sterols by precipitation with digitonin (Dam, 1928).

RESULTS AND DISCUSSION

Seasonal variations in composition of liver and of pyloric-caecal oils

Liver. A comparison of the results (Figs. 1 and 2) for the period September–October with those for the remaining 2-monthly periods showed, apart from the percentage vitamin A and percentage unsaponifiable matter in January–February and the liver weight values in March–April, highly significant differences ($P \leqslant 0{\cdot}01$). During September–October, the marked increase in the percentage vitamin A, sterol, total unsaponifiable matter and lipid phosphorus of the liver oil, as well as the rise in iodine value, is accompanied by a reduction in oil content and in liver weight. The reduction in liver weight is greater than would be anticipated from the lowering in oil content indicating the possible loss of components other than oil.

Pyloric caeca. The changes in oil content of the pyloric caeca (Fig. 2) resemble generally those of the liver with a highly significant decrease ($P \leqslant 0{\cdot}01$) in September–October as compared with the remaining 2-monthly periods. The pyloric-caecal and liver oils both show a significant reduction ($P \leqslant 0{\cdot}05$) in vitamin A content during July–August as compared with the remaining 2-monthly periods. Following a reduction in oil content in July–August the vitamin A content of the pyloric-caecal oil during September–October is merely restored to the normal value observed during the remainder of the year. In the liver, by contrast, there is a spectacular increase in vitamin A content associated with the reduced oil content at this period.

Seasonal changes in the vitamin A content of fish-liver oils (cf. Drummond & Hilditch, 1930) have been attributed to the effects of spawning, whereby the oil (triglyceride) is removed preferentially as compared with vitamin A leaving the residual oil in the liver enriched in this constituent. During the months of May, June and July, the groper are heavy in roe, and in the middle of August the catches fall off markedly, but increase again towards the end of the month. This suggests that seasonal changes in the vitamin A content are associated with spawning which, to judge from the size of the gonads, imposes a severe strain on the fish. The present results show also that apart from vitamin A, other constituents, including total unsaponifiable matter, cholesterol and probably vitamin D (cf. Weeber, 1945), tend to remain in the liver during mobilization of triglycerides after spawning.

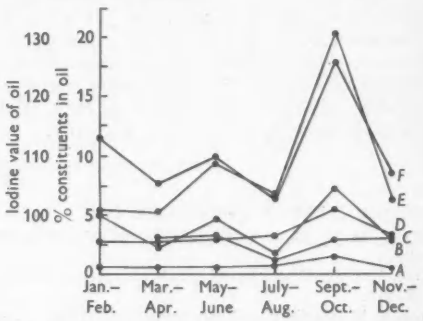

Fig. 1. Seasonal changes in: (*A*), % P in liver oil; (*B*), % vitamin A in liver oil; (*C*), % vitamin A in pyloric-caecal oil; (*D*), % cholesterol in liver oil; (*E*), iodine value in liver oil; and (*F*), % total unsaponifiable matter in liver oil.

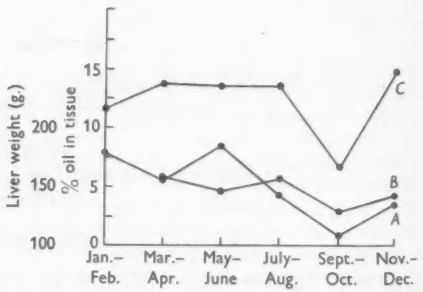

Fig. 2. Seasonal changes in: (*A*), liver weight; (*B*), % oil in pyloric caeca; and (*C*), % oil in livers.

Although the evidence of this work, as well as that of previous investigators (see Shorland, 1938), is consistent with the view that the increased vitamin A content of the liver oil is associated with a reduction in oil content following spawning, there is evidence in the present work that at other times

vitamin A may be preferentially mobilized or utilized while the proportion of triglycerides in the tissues remains unchanged. During the period July–August as compared with May–June, for example, there is significant decrease in vitamin A content of both the liver oil and of the pyloric-caecal oil without any significant change in the oil content of the tissues.

Investigations by Rapson & Schwartz (1944) on the South African stonebass (*Polyprion americanus*), which is closely related to the New Zealand groper, show that the liver of this species yields more oil (10–25%) than the groper, and that this oil is generally richer in vitamin A (2·4–25·9%) and in unsaponifiable matter (5·1–38·5%). The vitamin A content of the stonebass is at its highest during the period September–December and remains at a relatively low level during the autumn and winter months when the oil content tends to increase. The changes in vitamin A and oil content of the stonebass are thus generally more gradual and less pronounced than in the case of the groper. The high phospholipid content (5·86–14·6%) of stonebass-liver oil is paralleled in the present work on groper-liver oil where the mean 2-monthly values for the percentage phospholipid (calculated as percentage $P \times 100/3{\cdot}9$) range from 12·1 to 35·6, and generally indicate an inverse relationship between the yield of oil and its phospholipid content.

The present results accord with those of Rapson & Schwartz (1944) for stonebass-liver oil and with those of Haines & Drummond (1934) for halibut-liver oil in showing a more or less linear relationship between iodine value and vitamin A content.

During September, when the great majority of fish have spawned, two specimens about to spawn were selected for the purpose of studying the distribution of vitamin A and oil, as shown in

Table 1. *Distribution of oil and of vitamin A in the tissues of the groper*

	Male fish, total wt. 6852 g., caught 11. ix. 40					Female fish, total wt. 14975 g., caught 11. ix. 40				
Organ	Organ wt. (as % total weight of fish)	Oil (% of organ wt.)	Oil (as % total oil in fish)	Vitamin A (% of oil)	Vitamin A (as % of total vitamin A)	Organ wt. (as % total weight of fish)	Oil (% of organ wt.)	Oil (as % total oil in fish)	Vitamin A (% in oil)	Vitamin A (as % of total vitamin A)
Body (excluding backbone)	65·87	1·70	34·7	0·03	2·3	58·05	4·20	32·6	Trace	—
Head	23·92	6·93	51·4	Not found	—	22·10	14·90	43·9	Not found	—
Liver	1·34	6·45	2·8	14·1	88·9	2·25	8·19	2·4	11·1	93·5
Pyloric caeca	1·02	2·56	0·9	4·3	8·7	1·31	4·60	0·8	2·2	6·2
Intestines	0·77	1·58	0·3	0·2	0·1	0·89	2·73	0·3	0·3	0·3
Stomach	1·68	1·33	0·6	Not found	—	2·90	1·80	0·7	Not found	—
Milt (or roe)	1·18	1·92	0·6	0·03	Trace	9·27	11·34	14·0	Not found	—
Heart	0·19	1·41	Trace	Not found	—	0·19	4·28	—	Not found	—
Spleen	0·15	2·70	Trace	Trace	Trace	0·23	3·70	—	Trace	—
Backbone	3·88	7·10	8·7	Not found	—	2·81	14·34	5·3	Not found	—

Table 2. *Oil and vitamin A content in the livers as compared with those in pyloric caeca and intestines*

(For each material, five male and five female samples. All results as mean ± standard error of the mean.)

Organ	Weight of organ (g.)	Oil (%)	Vitamin A (%)
Male			
Livers	106·2 ± 13·6	9·08 ± 3·06	6·17 ± 1·73
Pyloric caeca	80·4 ± 7·7	4·76 ± 0·76	3·05 ± 0·83
Intestines	36·3 ± 8·1	5·10 ± 2·23	0·49 ± 0·22
Female			
Livers	162·7 ± 32·2	10·78 ± 2·61	3·33 ± 1·26
Pyloric caeca	104·5 ± 23·2	4·35 ± 0·69	1·97 ± 0·53
Intestines	49·0 ± 38·5	5·39 ± 1·00	0·21 ± 0·10

Table 1. The oil is widely distributed throughout the various tissues with the head, backbone, liver and roe forming the main depots. Most of the vitamin A is stored in the liver, but some is also found in the pyloric caeca, in addition to small proportions in the intestines; in the male specimen some vitamin A is also found in the body oil.

Table 2 shows results for the vitamin A and oil content of the livers, pyloric caeca and intestines from batches of fish sampled at intervals throughout the season. It will be seen that the oil content of the livers is approximately twice that of the pyloric caeca and intestines, while the vitamin A content of the liver oil is twice that of the pyloric-caecal oil and 12 to 13 times that of the intestinal oil.

Effect of size of liver on the percentage oil and its composition

The results of various investigators (cf. Drummond & Hilditch, 1930; Lovern, Edisbury & Morton, 1933; Pugsley, 1939) show that the vitamin A content of the liver oil increases with the size of the liver. In the case of the New Zealand ling (*Genypterus blacodes*) (Shorland, 1938) and the Cape ling (*G. capensis*) (Rapson, Schwartz & van Rensburg, 1944), however, there is a slight decrease in the vitamin A potency of the liver oil with size of liver, but owing to the probable increase in the proportion of liver in the larger fish, it is likely that the vitamin A reserves increase proportionately with the size of the fish.

Table 3 shows the results for small and large livers and the significance of the differences ascertained by pairing (Paterson, 1939) the large and small livers within each batch. It will be seen that the large livers, as compared with the small, contain approximately double the percentage oil, but the proportions of vitamin A, cholesterol, total unsaponifiable matter and phospholipid phosphorus in the oil are only about half those found in the case of the smaller livers. This means that the proportions of these constituents in the liver do not alter appreciably with the age of the fish, although the larger livers, as compared with the smaller, show a slightly increased content of total unsaponifiable matter and of cholesterol.

The livers from the male fish are smaller than those of the female, but the oil which was present in smaller proportions appears to contain more vitamin A, cholesterol and total unsaponifiable matter. The observations, however, were too few to establish any statistical significance in the differences observed.

SUMMARY

1. The percentages of vitamin A, cholesterol, total unsaponifiable matter and phospholipid, as well as the iodine value of the liver oil of the groper (*Polyprion oxygeneios*), have been shown to increase significantly immediately following spawning when the oil content of the liver is reduced.

2. The results are consistent with the view that the oil reserves of the liver are mobilized after spawning, while the reserves of vitamin A, cholesterol, total unsaponifiable matter and phospholipids are relatively unimpaired, leaving the remaining liver oil enriched in these constituents.

3. The pyloric caeca showed a similar reduction in oil content to that of the livers during September–October, but there was no spectacular rise in vitamin A content as observed in the case of the liver oils at this period.

4. In both the pyloric caeca and the liver there was a significant reduction in vitamin A content during

Table 3. *Effect of size of liver on the percentage oil and on the composition of the oil*

(All results as mean ± standard error of the mean. Significance of results is based on Student's technique (cf. Paterson, 1939) using nine pairs of samples, each pair representative of small and large livers from the same catch. HS ($P \leqslant 0{\cdot}01$), S ($P > 0{\cdot}01$ but $\leqslant 0{\cdot}05$).)

	Liver wt. (g.)	Oil (%)	Vitamin A	Cholesterol	Total unsaponifiable material	Phosphorus
				(as % of oil)		
Small	108·0 ± 8·87	9·67 ± 1·21	3·09 ± 0·60	3·98 ± 0·48	9·79 ± 1·34	0·86 ± 0·30
Large	214·0 ± 19·3	18·61 ± 2·38	1·76 ± 0·27	2·52 ± 0·25	6·12 ± 0·38	0·38 ± 0·05
Significance of differences	HS	HS	S	S	HS	HS

Table 4. *Effect of sex on the percentage oil in the liver and on the composition of the liver oil*

(Five samples of male and five of female material containing each 7–30 livers. All results as mean ± standard error of the mean. All differences not significant $P > 0{\cdot}05$.)

	Liver wt. (g.)	Oil (%)	Vitamin A	Cholesterol (as % of oil)	Total unsaponifiable material
Male	106·2 ± 13·6	8·11 ± 2·57	6·17 ± 1·74	4·75 ± 0·62	12·49 ± 3·01
Female	162·7 ± 39·5	11·23 ± 2·07	3·33 ± 1·13	3·79 ± 0·83	10·51 ± 2·91

July–August without any appreciable change in oil content suggesting the possibility of preferential removal of vitamin A as compared with the oil.

5. Studies on the distribution of vitamin A and of oil showed that whereas the oil was diffused more or less evenly throughout the fish, the vitamin A reserves are concentrated particularly in the liver, with somewhat smaller proportions in the pyloric caeca and in the body (male fish only).

6. It was found that although the percentage of oil increased with increasing size of liver, the contents of vitamin A, cholesterol, total unsaponifiable matter and lipid phosphorus in the liver were relatively unaffected.

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Efficiency of Oxidative Phosphorylation During the Oxidation of Pyruvate

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Krebs, Ruffo, Johnson, Eggleston & Hems (1953) have recently measured the value of the ratio

$$\frac{\text{equivalents of organic phosphate formed}}{\text{atoms of oxygen consumed}}$$

('phosphorylation quotient') for the reactions α-ketoglutarate → succinate and succinate → fumarate plus malate. The rate of formation of organic phosphate was determined by measuring the rate of incorporation of inorganic $^{32}PO_4$ into adenosinetriphosphate (ATP). The present paper is concerned with measurements, by the same procedure, of the phosphorylation quotient for the reaction pyruvate → acetate and for the complete oxidation of pyruvate.

EXPERIMENTAL

Preliminary experiments on liver and muscle suspensions prepared according to Krebs *et al.* (1953) showed that pyruvate, unlike the intermediates of the tricarboxylic acid cycle, did not maintain ATP in the suspensions. A more suitable material for the study of the oxidative phosphorylation associated with the oxidation of pyruvate was a suspension of washed subcellular particles of sheep kidney cortex, reinforced by cofactors, as previously described (Bartley, 1953). These were used in the present investigation. They were prepared from sheep kidneys within 3 hr. of collection and were used immediately. On keeping the tissue for 24 hr. at 0° in a mixture of frozen and liquid 0·9% (w/v) KCl the ability to carry out oxidative phosphorylation was reduced, although respiration remained unimpaired.

If the rate of incorporation of orthophosphate into ATP is to be maximal, ADP, as phosphate acceptor, must continuously be generated. The continuing formation of ADP from ATP can be brought about by the endogenous phosphatases, but, as shown by Potter & Recknagel (1951), the phosphatase activity of mitochondria separated in sucrose solutions is low. Active phosphatase may be liberated from the mitochondria by the action of KCl as shown by Berthet & de Duve (1951), or by the addition of nuclei as shown by Potter, Lyle & Schneider (1951). For these reasons the 'cyclophorase' type of preparation containing nuclei was

chosen. Unless otherwise stated, the 'R_3 residues' were used (Bartley, 1953). They were diluted with 0·9% (w/v) KCl to give about 70 mg. dry wt./2·0 ml. of suspension.

This type of subcellular preparation, made in a Waring Blendor, was satisfactory in promoting the turnover of ATP but is subject to a number of disadvantages; firstly the ability of the mitochondria to phosphorylate may be impaired; secondly the nuclei and some 'microsomes' that are mixed with the mitochondria can contribute to the O_2 uptake without concomitant phosphorylation. For these reasons the phosphorylation quotients obtained are probably somewhat too low.

The suspensions were incubated at 20° in a series of conical manometric Warburg vessels, usually ten, which contained the same mixture, but were incubated for different periods. Before the suspensions were prepared the following solutions were measured into the main compartments of the vessels: 0·5 ml. 0·02 M-sodium ATP, 0·2 ml. 0·1 M-phosphate buffer pH 7·4, 0·2 ml. 0·1 M-$NaHCO_3$, 0·2 ml. 0·02 M-$MgSO_4$, 0·1 ml. 0·1 M-sodium pyruvate, 0·2 ml. $1{\cdot}86 \times 10^{-4}$ M-cytochrome and 0·5 ml. water. The side arm contained 0·1 ml. of a solution of $KH_2{}^{32}PO_4$ in 0·9% (w/v) KCl, the centre well 0·2 ml. 2 N-NaOH and filter paper. The vessels were kept in an ice-water mixture until the suspension was ready, and 2 ml. of the suspension (R_3 residue in 3 parts 0·9% (w/v) KCl) were measured into each vessel; 0·5 ml. 30% (w/v) trichloroacetic acid was added to one sample to stop enzymic activities, and the contents of the side arm were tipped in to give the zero value. The other cups were attached to the manometers, quickly gassed with O_2 and placed in a rack in such a way that the vessels were immersed in a trough of ice water. At zero time the contents of the main compartment and the side bulb of the first vessel were mixed and the manometer was shaken in the water bath at 20°. This procedure was repeated with the rest of the vessels at 30 sec. intervals. The first manometer was taken from the bath after 2 min. incubation, the vessel was removed from its manometer, placed in a bath of ice and water and 0·5 ml. 30% (w/v) trichloroacetic acid was added. The procedure was repeated with other vessels after 3, 4, 6, 8, 10, 20, 30 and 40 min. incubation. The contents of the cups were centrifuged for 10 min. at 0° and the supernatant was stored at −14° until it could be analysed. The trichloroacetic acid extract was used for the quantitative determination of orthophosphate, ATP and pyruvate and for the measurement of the radioactivity of the phosphate fractions. The O_2 uptake was measured in the vessels incubated for 20, 30 and 40 min. The average rates were used in the calculation of the phosphorylation quotient.

Separation and estimation of the phosphate fractions. As the added inorganic orthophosphate and ATP constituted more than 95% of the total phosphate of the trichloroacetic acid extracts, other phosphates could be ignored. The separation of inorganic phosphate and of ATP was effected by ascending paper chromatography using the formic acid-*iso*propyl ether mixture of Hanes & Isherwood (1949). The papers, Whatman no. 1, 45 × 19 cm., were washed in a solution of ethylenediaminetetraacetic acid as described by Eggleston & Hems (1952) and 0·125 ml. of the solution to be chromatographed was placed with an Agla syringe (Burroughs Wellcome Ltd.) along a continuous line 5 cm. long at the start of the chromatogram. It was dried by a current of warm air from a hairdrier. The chromatograms were developed from 2 to 4 hr. After drying the bands were located by the molybdate spray of Hanes & Isherwood (1949). In some experiments ethyl ether was used instead of *iso*propyl ether in the solvent mixture, because with this solvent separation of inorganic orthophosphate from adenosinepolyphosphates could be achieved in 30 min.

The blue bands corresponding to the ATP and the inorganic orthophosphate, usually about 7 × 3 cm. were cut out from the paper and put into test tubes graduated at 10 ml., together with 2·5 ml. of the ashing fluid of Hanes & Isherwood (1949). The paper strips were wet ashed, the volume was made up to the mark with water, and after

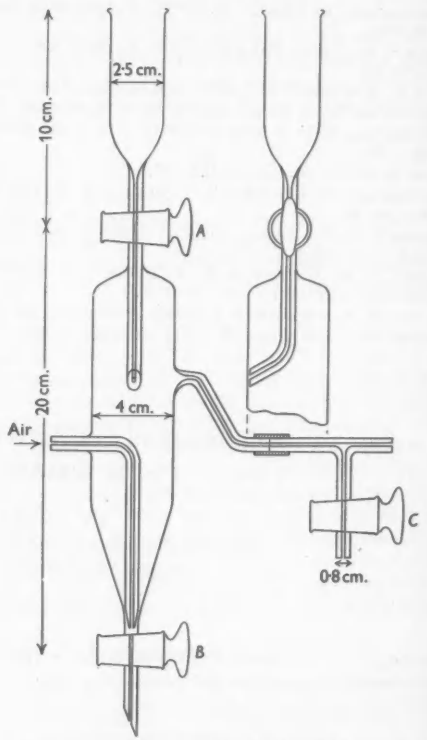

Fig. 1. Funnels used in the serial determination of phosphates by the method of Berenblum & Chain (1938). Taps *A* and *C* are opened to allow addition of reagents: all taps are shut when air is blown through: taps *C* and *B* are open when liquid is run off.

mixing 2 ml. samples of the fluid were analysed for phosphate and radioactivity. The paper area between the adenosinepolyphosphates and the inorganic orthophosphate was similarly ashed for determination of the radioactivity of other organic phosphates between these areas. A measured area of about 7 × 4 cm. was cut from the front of the paper and analysed for the phosphate content of the paper.

To test for the presence of phosphate compounds other than adenosinepolyphosphates and inorganic orthophosphate 25 μl. samples of the filtrate were developed by descending paper chromatography with the *p*-toluene-

sulphonic-*tert.*-amyl alcohol-water mixture of Hanes & Isherwood (1949). After 17 hr. all the phosphate compounds tested (ATP and ADP, adenylic acid, thiaminediphosphate, thiaminemonophosphate, hexosediphosphate, pyrophosphate, orthophosphate, phosphopyruvate, phosphoglyceric acid, pyridoxalphosphate) were separated, except ATP and ADP which travelled together. The spots were located with the molybdate spray, cut out and analysed as described. No detectable amounts of phosphate other than (ATP and ADP) or orthophosphate were found in the suspensions of tissue particles.

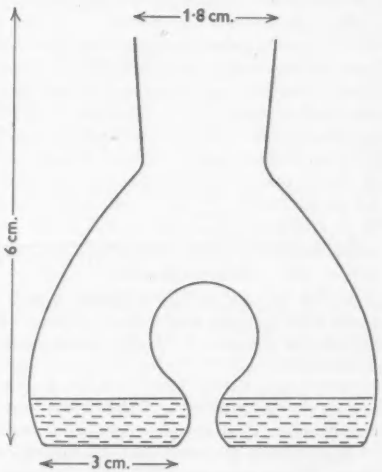

Fig. 2. Modified Warburg vessel used for the estimation of pyruvate by carboxylase in the presence of trichloroacetic acid (p. 679) when carboxylase and test solutions were approx. the same volume, 3–5 ml.

The phosphate content of the wet-ashed material was estimated by the method of Berenblum & Chain (1938). The use of a series of funnels of the shape shown in Fig. 1 was found useful when large numbers of estimations were required. The *iso*butanol layer was run off after the second sulphuric acid washing and was stored together with the ethanol washings of the funnel until they could be analysed in the spectrophotometer. The *iso*butanol extracts showed no change after 3–4 days' storage. Immediately before the reading was taken the colour was developed by the addition of 0·1 ml. of the stannous chloride solution (10 g. in 25 ml. conc. HCl). The solution was made up to volume with pure absolute ethanol, mixed, and the colour was read in the Beckman spectrophotometer at 750 mμ. No redistillation of the *iso*butanol (commercial grade containing 95% *iso*-butanol) and ethanol used was required as the green colour described by Eggleston & Hems (1952) did not occur.

Radioactivity measurements were carried out on 2 ml. samples of the paper digests diluted to 10 ml. A β counting tube, liquid type M6 (20th Century Electronics) was used.

Pyruvic acid. This was estimated either by the specific extraction method of Friedmann & Haugen (1943) or by the carboxylase method of Krebs & Johnson (1937), which was slightly modified because the solutions to be analysed contained trichloroacetic acid. The trichloroacetic acid extract (1 ml.) was put in one arm of a manometer vessel of the type shown in Fig. 2. To it was added 0·1 ml. of aqueous 0·02% (w/v) bromocresol green and enough N-NaOH to bring the pH to about 4·7. The total volume was brought to 5·0 ml. with water. In the other arm of the vessel was put 3·0 ml. yeast carboxylase, which had been brought to pH 4·7 with 3M-acetate buffer and stored at −14° for at least 2 weeks. Yeast carboxylase at this pH may be stored at −14° for at least 9 months without loss of activity. After about the first fortnight of cold storage the blank CO_2 output of the carboxylase becomes negligible. The gas output after mixing was usually complete after about 60 min. Glucose did not interfere. Colorimetric and manometric methods agreed to within 5%.

Special chemicals. Barium ATP was prepared according to LePage (1949) and the preparation of the sodium salt and the estimation of its purity was carried out by the method of Eggleston & Hems (1952); 86% of the total P in the sample was present as ATP and 11% as ADP.

Pyruvic acid was redistilled from a commercial sample. The sodium pyruvate used as a standard in the colorimetric method was a pure sample kindly supplied by Dr S. R. Elsden.

RESULTS

Phosphorylation quotient during complete oxidation of pyruvate. As previously described (Bartley, 1953), complete oxidation of pyruvate to carbon dioxide and water takes place in suspensions of washed sheep-kidney particles when the pyruvate concentration is low (0·0025M), sodium bicarbonate (0·075M) is present, and an amount of the particles equivalent to 70–80 mg. dry wt. is used in 4 ml. The concentrations of ATP and inorganic phosphate and the distribution of radioactivity after various periods of incubation under these conditions are shown in Table 1. The data indicate that the initial concentration of ATP was maintained and that incorporation of radioactivity into the ATP occurred. The rate of incorporation v, calculated by the formula of Krebs *et al.* (1953), was reasonably constant for the initial period (8 min.) and subsequently dropped. The phosphorylation quotient for the initial period was about 2·2. In three similar experiments the initial quotient was between 2·1 and 2·6, average 2·2. The addition of 0·00005M-cocarboxylase lowered the phosphorylation quotient to 1·6.

Phosphorylation quotient for the reaction pyruvate → acetate. On reduction of the bicarbonate concentration, or increase of the pyruvate concentration above 0·0025M, the suspension oxidizes pyruvate incompletely. The ratio O_2 absorbed/pyruvate removed (O_2/pyruvate ratio) falls and acetate appears as an end product (Bartley, 1953). A series of measurements of the rates of incorporation of radioactivity into the added ATP was carried out under conditions when the O_2/pyruvate ratio varied between 0·5 and 1·5. The phosphorylation

Table 1. *Incorporation of radioactivity into ATP during the oxidation of pyruvate by a sheep-kidney suspension*

(For details see text. Oxygen uptake 0·894 μg. atom/min. Ratio O_2/pyruvate was 2·5 throughout.)

Time of incubation (min.)	Orthophosphate* in vessel $= a$ (μmoles)	Labile P* of ATP in vessel $= b$ (μmoles)	Radioactivity in chromatogram fractions (% of total)			Ratio: Specific activity of labile P of ATP / Specific activity of orthophosphate	Rate of exchange of P† $= v$ (μmoles/min.)	Ratio: P exchanged / atom O_2 uptake
			Orthophosphate $= x$	ATP	Rest of the paper			
0	22·80	11·34	100·00	0	0	0	0	0
2	28·00	11·22	89·00	10·54	0·46	0·242	1·882	2·15
3	27·00	11·30	84·20	15·50	0·30	0·441	2·02	2·24
4	27·04	11·90	79·60	20·20	0·20	0·577	2·37	2·66
6	24·70	11·78	75·20	24·27	0·53	0·681	1·94	2·17
8	24·62	12·20	72·10	27·40	0·50	0·767	1·86	2·08
10	24·88	11·20	73·40	25·43	1·17	0·776	1·53	1·70
20	25·00	14·32	67·30	31·90	0·80	0·830	1·096	1·22
30	22·64	13·30	66·70	31·90	1·40	0·882	0·829	0·92
60	26·28	13·16	65·70	32·86	1·44	1·000	—	—

* Phosphates are calculated from the amounts recovered from the paper chromatogram. The quantities of ATP, orthophosphate and radioactivity added to each vessel were only approximately equal.

† Calc. from eqn. $v = \frac{ab}{t(a+b)} \ln \frac{bx_0}{x(a+b) - ax_0}$.

quotients calculated from these experiments ranged from 2·35 to 3·92. In Fig. 3 all the phosphorylation quotients obtained are plotted against the corresponding O_2/pyruvate ratio. The regression line for the points was calculated. This gave a phosphorylation quotient of 4·06 (S.E. 0·4) when the O_2/pyruvate ratio was 0·5, i.e. when pyruvate was quantitatively converted to acetate.

Fig. 3. Plot of phosphorylation quotient against O_2/pyruvate when pyruvate was oxidized by sheep kidney suspensions in presence of varying concentrations of bicarbonate.

Phosphorylation quotient with fumarate or oxaloacetate as catalyst. With the above method no values for the O_2/pyruvate ratio between 1·5 and 2·5 were obtained. When bicarbonate was replaced by catalytic amounts of fumarate the required high concentration of ATP was not maintained in three out of four experiments. In the fourth experiment, with 0·0025 M-pyruvate and 0·001 M-fumarate, ATP was maintained and the resulting O_2/pyruvate ratio was 1·7; the phosphorylation quotient was 1·02.

ATP was also maintained in the presence of 0·001 M-oxaloacetate and 0·0025 M-pyruvate. The phosphorylation quotient with these substrates ranged from 1·7 to 2·49 (mean value of 2·13), whilst the O_2/pyruvate ratio varied between 0·7 and 1·3. Thus neither oxaloacetate nor fumarate was as efficient as bicarbonate in promoting complete oxidation; both fumarate and oxaloacetate depress the value of the phosphorylation quotient. The low O_2/pyruvate ratio in the presence of oxaloacetate, compared with that in presence of sodium bicarbonate, is probably due to lack of penetration of oxaloacetate into the mitochondria (Bartley & Davies, 1952). The same authors showed that fumarate at the concentration used was concentrated in the particulate fraction of the mixtures in the proportion of 8 to 1, whereas pyruvate was approximately evenly distributed between the particulate and the soluble fractions. Bicarbonate distribution was not measured, but it seems unlikely that a

barrier would exist to the free diffusion of carbon dioxide. These results may explain the inability of oxaloacetate to replace bicarbonate completely in the oxidation of pyruvate to carbon dioxide and water.

Phosphorylation at pH 7·8. At pH 7·0 pyruvate in concentrations above 0·0005 M is oxidized almost quantitatively to acetate, provided that bicarbonate or intermediates of the tricarboxylic acid cycle are not added, the O_2/pyruvate ratio being 0·5. At a higher pH or lower pyruvate concentrations some complete oxidation occurs without the addition of bicarbonate or other catalysts (Bartley, 1953).

The study of oxidative phosphorylation at pH 7·8 showed the formation of a phosphate compound running on the paper chromatogram between ATP and orthophosphate when a *p*-toluenesulphonic acid-*tert.*-amyl alcohol-water mixture was used as the solvent; the specific activity was about 50 % of that of ATP-phosphorus. The identity of the substance has not been definitely established but the position on the chromatogram suggests that it is pyrophosphate. Pyrophosphate was never detected in experiments at lower pH when the O_2/pyruvate ratio was of the same order (0·87) as that obtained at pH 7·8. This is in agreement with the findings of Lehninger & Smith (1949) and Cross, Taggert, Covo & Green (1949) that pyrophosphate did not accumulate at pH 7·4 in the absence of fluoride. The accumulation of a third phosphate made the formula of Krebs *et al.* (1953) inapplicable.

Phosphorylation in unfractionated suspensions of subcellular particles. To investigate whether the fractionation of the suspensions by centrifugations and washing damages the mechanism of phosphorylation, experiments were carried out with unfractionated suspensions of subcellular particles made from 50 g. of kidney cortex with 200 ml. of 0·9 % (w/v) potassium chloride. 2·0 ml. of this preparation were diluted with 2 ml. substrate solution. Rapid incorporation of radioactivity into the ATP fraction was obtained. No breakdown of ATP occurred and the oxygen uptake was linear with time; the calculated average phosphorylation quotient was 1·3. The oxygen uptake was much greater than that required for the complete oxidation of the pyruvate removed, the O_2/pyruvate ratio being approximately 5. Thus substrates other than pyruvate were oxidized.

When the suspension was diluted with an equal volume of 0·9 % (w/v) potassium chloride the oxygen uptake was less than required for the complete oxidation of the pyruvate removed, the O_2/pyruvate ratio being 2·0.

Incubation of an unfractionated suspension without added substrate but with the other standard additions showed that ATP was not maintained under these conditions. During the first 10–15 min. of incubation the ATP concentration fell about 20 %. An approximate estimate of the phosphorylation quotient, ignoring the fall of the ATP concentration, gave a value of 0·82 (range 0·63–1·04).

As the oxygen uptake during the oxidation of pyruvate by unfractionated suspensions was greater than was required for the complete oxidation of the pyruvate removed, the distribution of oxidative capacity between the particulate and liquid fractions of a kidney suspension was measured. The suspension was centrifuged at 2000 *g* for 15 min. to sediment the particles, and the supernatant was poured off. The measurement of oxygen consumption indicated that 54 % of the total oxygen uptake was associated with the particulate fraction. As the ability to carry out oxidative phosphorylation is associated with the particles in the suspension (Green, Atchley, Nordman & Tepley, 1949; Still & Kaplan, 1950; Harman, 1950; Green, 1951; Harman & Feigelsen, 1952), it is only their oxygen consumption that is relevant to the phosphorylation quotient. Recalculation of the phosphorylation quotient for the whole suspensions on this basis gave a value of 2·42 for the complete oxidation of pyruvate and 1·52 for the endogenous substrate. The value of 2·42 is in good agreement with the results obtained with the washed suspensions. The value of 1·52 obtained when no substrate was added to the suspension would indicate that endogenous respiration does not originate from the oxidation of pyruvate alone.

DISCUSSION

A phosphorylation quotient of 4 for the oxidation of pyruvate to acetate is in agreement with the value postulated for the analogous oxidative decarboxylation of α-ketoglutarate (Krebs *et al.* 1953; Hunter & Hixon, 1949).

Previous values for the phosphorylation quotient with pyruvate as substrate vary between 2·5 (Cross *et al.* 1949) and about 3 (Lardy & Wellman, 1952; Ochoa, 1943). Cross *et al.* and Lardy did not measure the amount of pyruvate oxidized, and it is uncertain whether pyruvate was completely oxidized. In Ochoa's experiment the O_2/pyruvate ratio was 2·5, indicating complete oxidation. The phosphorylation quotient measured was 1·9, and a value of 3·1 was arrived at by making corrections of questionable validity for dephosphorylation.

A phosphorylation quotient of 3 is expected for the complete oxidation of pyruvate (see Ochoa & Stern, 1952). The reason for the lower values of 2·1–2·6 obtained in the present experiments cannot be satisfactorily explained. It is likely that the observed value does not represent the efficiency in the intact tissues, and that some efficiency is lost by the treatment of the material.

SUMMARY

1. The phosphorylation quotient

$$\left(\frac{\text{equivalents of organic phosphate formed}}{\text{atoms of oxygen consumed}}\right)$$

associated with the oxidation of pyruvate by suspensions of washed subcellular particles of kidney cortex was calculated from measurements of the rate of incorporation of ^{32}P into adenosinetriphosphate.

2. When pyruvate, in the presence of bicarbonate, was completely oxidized (as indicated by an O_2/pyruvate ratio of 2·5) the average phosphorylation quotient was 2·23 (range 2·08–2·6).

3. When pyruvate was incompletely oxidized phosphorylation quotients up to 3·92 were obtained. From the regression line obtained by plotting phosphorylation quotients against the O_2/pyruvate ratio, a phosphorylation quotient of 4 was obtained for the oxidation of pyruvate to acetate.

4. Either bicarbonate, oxaloacetate or a precursor of oxaloacetate must be present if pyruvate is to be oxidized beyond acetate. The average phosphorylation quotient in the presence of fumarate (1·7) or oxaloacetate (2·13) was lower than that obtained with bicarbonate. Unfractionated suspensions completely oxidizing pyruvate gave a phosphorylation quotient of 1·3. Reasons for phosphorylation quotients below the expected value of 3 are considered.

5. In presence of 0·001 M-fumarate or oxaloacetate, pyruvate (0·0025 M) was oxidized beyond the stage of acetate (O_2/pyruvate ratio greater than 0·5) but was not completely oxidized.

6. At pH 7·8, a phosphorus-containing compound other than adenosinetriphosphate or orthophosphate, probably pyrophosphate, was formed.

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Changes in Lens During the Formation of X-ray Cataract in Rabbits

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Experimental work on cataract is directed towards finding some change which precedes or occurs early in opacity formation. Work has been hampered by lack of suitable experimental material, and though there is considerable information about the end results of senile cataract (Bellows, 1944), little is known of the changes occurring in animal lenses during the production of experimental cataract. The chemistry of the two lenses of a single normal animal seems to be very similar (Bellows, 1944), just as their weights are practically identical. It is therefore an advantage to be able to produce cataract in one lens only, leaving the second as a control. We have chosen to irradiate the right eye of rabbits and thus produce a cataract in this eye while barely affecting the other. Irradiation

cataract due to X-rays has been known for a long time and has been described in detail in rabbits by Poppe (1942), von Sallmann (1951), Cogan & Donaldson (1951) and others. Standardization of age of rabbit and technique of irradiation and dosage leads to a predictable course of opacity formation. In our experiments the lens is usually opaque and intumescent about 7 months after irradiation, and the metabolism of the right lens can be directly compared with that of the left lens. Such a comparison may be made at any stage after irradiation.

We have compared the activities of five enzymes in such lenses: (i) glutathione (GSSG) reductase, so far the only enzyme in lens known to use GSSG as substrate; (ii) and (iii) malic enzyme and *iso*citric dehydrogenase, both of which can be coupled in lens with the GSSG-reductase through triphosphopyridine nucleotide (TPN) (van Heyningen & Pirie, 1953); (iv) lactic dehydrogenase; and (v) cytochrome *c* reductase. Cytochrome *c* has been shown to be present only in the epithelium of the lens (Ely, 1952) and the function of this enzyme throughout the substance of the lens is not known.

Decrease of SH groups in senile cataract is well established, and diminution of the ascorbic acid in the aqueous humour has also been reported (see Bellows, 1944). We have therefore studied the effect of irradiation on the reduced glutathione (GSH) and protein SH (PSH) of the lens and on the ascorbic acid of the aqueous humour, vitreous humour and, where possible, the lens.

EXPERIMENTAL

Animals. Dutch rabbits 6±2 months old were used. Nearly all were 6 months old.

Clinical examination. The eyes were examined with the slit-lamp and ophthalmoscope before irradiation and at various times afterwards in order to determine the course of cataract formation.

Irradiation. The irradiation was carried out with a Philips Metalix 200 kV. X-ray therapy set, operated at 190 kV., 6 ma. with a filter of 0·5 mm. Cu + 1 mm. Al. A lead tube 2·5 cm. int. diam. and 18 cm. long defined a vertical X-ray beam. The rabbit to be treated lay on its left side with the right eye centrally placed in the end of the lead tube. The distance from the X-ray target to the end of the lead tube was 28 cm. The dose rate at the level of the rabbit's lens was measured before each set of irradiations with four small condenser ionization chambers (BD2 chambers made from bakelite graphite and calibrated at the National Physical Laboratory) placed side by side in a Perspex block. On different occasions the dose rate varied from 67 r./min. to 72 r./min., but the exposure time was chosen to give a total dose of 1400 r. to the right lens in every case. Although the measurement of dose rate and of time were both accurate to 1%, variations in position of the rabbits might lead to errors of a few per cent in the dose to the lens, but certainly not as much as 5%. The dose received by the left lens during this treatment was measured by irradiating in the usual way a rabbit carcass from which the left eye had been removed and replaced by a condenser chamber enclosed in a thin waterproof sheath. It was found that the left eye, if on the axis of the beam, received a dose of 548–596 r. In setting up the rabbits, however, it was the position of the right eye which was considered important and the left eye was not always central in the X-ray beam. On many occasions, therefore, the left eye probably received much less than this. The position of the X-ray beam both at entry and at exit became visible on the rabbit as a patch of grey hair, and in many cases this showed that the left lens must have been outside the beam of X-rays.

Removal of eyes and preparation of eye tissues and fluids for examination. The rabbit was killed with intravenous Nembutal. The eyes were at once excised and blotted to remove blood. A capillary glass pipette was then pushed through the cornea, and the aqueous humour, which ran into the pipette, was blown out into a weighed tube containing 0·2 ml. 5% (w/v) metaphosphoric acid which was then reweighed. The weight of the aqueous humour was usually 0·1–0·2 g. The cornea and the iris were then cut off and the lens removed in its capsule by cutting round the zonule. Small tags of pigmented ciliary processes could be removed by blotting gently with filter paper. The vitreous body was next expressed from the back part of the eye, weighed and dropped into 2 ml. 5% (w/v) metaphosphoric acid. The weight of the vitreous body was between 1·0 and 2·0 g. The metaphosphoric acid solutions of the aqueous humour and vitreous body were centrifuged and the supernatants used for the estimation of ascorbic acid.

Lens suspension. The lens was weighed, chopped, and ground with a little sand and three times its weight of distilled water. The sand quickly settled and samples of the suspension were taken for estimation of GSH and some of the enzymes; the rest was dialysed 24–48 hr. against running tap water.

Lens extracts. The lens extracts were opalescent and were prepared by centrifuging the suspension for 20 min. at 7000 *g*.

Diphosphopyridine nucleotide (DPN) and TPN. A mixture of the crude nucleotides was prepared by the method of LePage & Mueller (1949).

o-Iodosobenzoic acid. This was prepared by the method of Askenasy & Meyer (1893).

Methods

Ascorbic acid. This was estimated by titration with 2:6-dichlorophenolindophenol made up so that 1·0 ml. ≡ 0·1 mg. ascorbic acid. Aqueous humour (0·05 ml.) was titrated with dye contained in an Agla micrometer syringe, using mechanical stirring of the drop. Vitreous humour was titrated from a 2·0 ml. burette using 0·2 ml. dye.

Reduced glutathione. 0·2 ml. undialysed lens suspension was precipitated with 1·5 ml. 2·5% (w/v) metaphosphoric acid and GSH was estimated in the extract by the method of Grunert & Phillips (1951).

Protein SH. This was estimated in undenatured lens protein by an adaptation of the method of Hellerman, Chinard & Deitz (1943). 1·0 ml. *o*-iodosobenzoate (approx. 0·005 N, standardized against 0·005 N-$Na_2S_2O_3$) was added to a suitable volume of dialysed lens extract or suspension (0·1–0·3 ml.) in 2·0 ml. 0·1 M-potassium phosphate buffer, pH 7. After exactly 2 min. a freshly prepared solution of 0·5 g. KI in 5 ml. 0·1 N-HCl was added, and the iodine liberated from the excess *o*-iodosobenzoate titrated against

0·005 N-$Na_2S_2O_3$. During the 2 min. incubation we found that the colour given by the nitroprusside reaction decreased from a bright puce to a very pale rose, but was not quite abolished. Further incubation decreased the colour only very slowly and at the same time the amount of *o*-iodosobenzoate reacting with the lens slowly increased. However, as groups in proteins other than SH may react with *o*-iodosobenzoate, the incubation time was restricted to the 2 min. suggested by Hellerman *et al.* (1943).

Malic enzyme. This was estimated by following the reduction of TPN as previously described (van Heyningen & Pirie, 1953).

iso*Citric dehydrogenase.* Reduction of TPN was followed in a Beckman spectrophotometer according to the method of Lotspeich & Peters (1951). 0·3 ml. was used and readings were taken at 1 min. intervals for 10 min.

Lactic dehydrogenase. Reduction of DPN was followed spectrophotometrically according to the method of Askonas (1951). 0·01 or 0·02 ml. undialysed lens extract was used and readings were taken at 0·5 min. intervals for 2·5 min.

Activity of the malic enzyme, and of *iso*citric and lactic dehydrogenases is expressed as increase in optical density ($\Delta \log I_0/I$) at 340 mμ./min. for the whole lens.

Cytochrome-c reductase. Reduction of cytochrome *c* was measured at 550 mμ. TPN was reduced in the Beckman cell using *iso*citric dehydrogenase in a dialysed extract of acetone heart powder (Lotspeich & Peters, 1951) which itself had no cytochrome reductase activity. The system consisted of D-*iso*citrate (1·4 μmoles), $MnCl_2$ (2·0 μmoles), TPN (approx. 0·28 μmole), heart extract (0·1 ml.), cytochrome *c* (prepared by the method of Keilin & Hartree, 1937) (0·2 ml.) and 0·029 M-veronal-acetate buffer (Michaelis, 1931), pH 7 (2·0 ml.); total vol. 2·95 ml. Reduction of TPN was followed at 340 mμ. until nearly complete (5 min.); 0·05 ml. undialysed lens extract was then added and the increase of absorption at 550 mμ. read at 1 min. intervals for 5 min. The activity is expressed as increase in optical density at 550 mμ./min. for the whole lens.

Glutathione reductase. The activity of this enzyme was measured by coupling it with the decarboxylation of *iso*citrate (Ochoa, Mehler & Kornberg, 1948), according to the following equations:

$$\textit{iso}\text{Citrate}^{3-} + \text{TPN}^{+} = \text{TPNH} + \alpha\text{-ketoglutarate}^{2-} + \text{CO}_2$$
$$\text{TPNH} + \text{GSSG} + \text{H}^{+} = 2\text{GSH} + \text{TPN}^{+},$$

where TPN^+ represents oxidized TPN and TPNH represents reduced TPN. Although lens will decarboxylate *iso*citrate, a dialysed extract of acetone heart powder (which we found contained no GSSG-reductase) was added to ensure that the reductase was always the limiting enzyme. Dialysed lens extract or suspension (0·5 ml.) was incubated at 37° with heart extract (0·1 ml.), GSSG (8·1 μmoles), *iso*citrate (9·4 μmoles), TPN (approx. 0·28 μmole), $MnCl_2$ (0·5 μmoles) and 0·029 M-veronal-acetate buffer pH 7·25 (0·8 ml.); total vol. 1·8 ml. A blank with all the reagents except lens was always included. One drop of $CHCl_3$ was added to each tube and 0·4 ml. samples were pipetted into 2·0 ml. freshly prepared cold 10% (w/v) trichloroacetic acid at intervals, usually 0, 1·5, 3 and 4·5 hr. α-Ketoglutarate was estimated in the extract by the method of Friedemann & Haugen (1943) and the activity of the enzyme was expressed as mg. α-ketoglutaric acid produced by the whole lens/hr. Under these conditions it was not possible to estimate the increase in GSH, owing to the uncatalysed interaction between GSH, GSSG and the SH and S.S groups in protein.

RESULTS

Clinical course of formation of lens opacity

This was very similar in all the rabbits. Within 24 hr. of irradiation both eyes showed cells in the anterior chamber with a slight flare and debris on the lens capsule. This quickly subsided and was followed in about a week by a cellular invasion of the anterior vitreous body, which might last for a week or a fortnight. About 3 weeks to 1 month after irradiation many rabbits showed a massive pigment slide on the cornea caused by wandering of the pigmented epithelium of the limbus on to the cornea. The fur came off the lids in 4–7 weeks and was later replaced by sparse grey fur. First signs of lens opacity occurred in about 4 months when small greyish opacities could be seen with the slit-lamp at the back of the lens. Water clefts at the equator and changes in the anterior cortex were evident in about 5 months and the opacity was often sufficiently dense to prevent view of the fundus 6 months after irradiation. We found, like Cogan & Donaldson (1951), that rate of opacity formation depended very much on the age of the rabbit when irradiated. The left lens is estimated to receive a maximum of about 40% of the radiation received by the right lens and the eye showed the cellular reactions in the anterior chamber and vitreous body. In a few rabbits there was a small granular opacity at the centre back surface of the left lens but at the time the rabbit was killed this involved only a minute part of the total lens.

The results from nineteen rabbits are arranged in Table 1, in which the rabbits are listed in decreasing order of the severity of their cataract, judged by slit-lamp examination on the day before death. The order of cataract severity was decided by one of us before knowing the order of the changes of lens constituents. The lens was opaque in nos. 1–8 inclusive. Nos. 9–13 showed major but not complete opacities. The last seven (nos. 14–20) were killed at intervals before the development of severe cataract, in order to find out at what stage metabolic changes could first be observed. Nos. 14–17, inclusive, had relatively slight opacities confined to the front and back of the lens. No. 18, killed 3 months after irradiation, showed no changes in the lens on slit-lamp examination. The lenses of nos. 19 and 20, killed 20 hr. after irradiation, showed no abnormality, although the usual initial inflammatory reactions were present in both eyes. In these young rabbits the two right lenses were combined and the two left lenses were combined, as a single lens was too small for all the determinations.

In no animal did the left lens, on removal, show any changes visible to the naked eye, although in some of the rabbits with severely affected right

lenses the slit-lamp appearance of the left lens had been slightly abnormal. Any abnormality in the left lens was certainly tiny and for simplicity we shall refer to the left lenses as normal.

It seems that the lens in the earlier stages of cataract weighed rather less than the normal lens (Table 1, rabbits nos. 9–17 inclusive). On the other hand, the severely cataractous lens was usually considerably heavier than the normal one, apparently because of an increase in water content (rabbits nos. 1–8 inclusive). To take into account this variation between the weights of the two lenses, comparisons are made throughout between whole lenses and not on a wet weight basis. This tends to minimize losses in the heavy cataract lenses but to maximize losses at the early stage.

Effect of irradiation on GSH and protein SH in lens

The GSH content of right and left lenses was compared on fifteen occasions (Table 1). In the eight rabbits in which the cataract was complete there was a decrease of GSH in the right lens of 84 % or more. In the rabbits in which only a small part of the lens was affected, the decreases ranged from 57 % in no. 14 to 20 % in no. 17. In general, the

Table 1. *Comparison between right (irradiated) and left lenses. Changes in reduced glutathione, protein SH and glutathione reductase*

(The rabbits are listed in order of decreasing severity of lens opacity. GSSG-reductase activity is given in mg. α-ketoglutaric acid formed by the whole lens/hr. Detailed methods are given in the Methods section.)

No.		Lens wt. (mg.)	Time after irradn. (months)	SH per lens (mg.) GSH	Loss (%)	PSH suspn.	Loss (%)	PSH extract	Loss (%)	GSSG-reductase Extract	Loss (%)
1	R	546	9	0·029	85	0·37	53	0·14	75	—	
	L	481		0·199		0·79		0·55		—	
2	R	726	5·5	—		—		—		0·042	48
	L	500		—		—		—		0·081	
3	R	595	5	0·037	84	—		—		—	
	L	490		0·232		—		—		—	
4	R	547	7	0·027	84	0·59	44	0·35	55	0·058	54
	L	502		0·166		1·06		0·77		0·125	
5	R	592	8	0·013	92	—		—		—	
	L	499		0·162		—		—		—	
6	R	495	6	—		0·68	39	0·50	45	—	
	L	486		—		1·12		0·91		—	
7	R	725	5·5	0·021	87	—		—		0·073	41
	L	514		0·165		—		—		0·123	
8	R	663	6	0*	>84	—		—		0·029	63
	L	473		0·131		—		—		0·079	
9	R	499	7	0·057	70	—		0·81	17	0·055	49
	L	527		0·188		—		0·98		0·109	
11	R	500	7	—		—		—		0·011	88
	L	518		—		—		—		0·089	
12	R	459	4·5	0·044	75	—		—		0·038	54
	L	498		0·175		—		—		0·082	
13	R	389	5	0·053	73	0·56	19	0·46	31	0·083	49
	L	496		0·195		0·69		0·67		0·162	
14	R	439	7·5	0·071	57	0·65	13	0·58	18	0·106	34
	L	509		0·167		0·75		0·71		0·161	
15	R	406	5	0·075	56	—		0·63	2	0·089	17
	L	430		0·172		—		0·64		0·107	
16	R	430	7·5	0·146	23	—		—		0·112	11
	L	499		0·189		—		—		0·126	
17	R	472	4·5	0·130	20	0·74	0	0·66	3	0·054	23
	L	491		0·162		0·74		0·68		0·070	
18	R	470	3	0·167	13	0·69	1	0·54	4	—	
	L	468		0·193		0·70		0·56		—	
19	R	400	20 hr.	0·158	4	0·56	0	0·40	2	0·034	0
20	L	412	20 hr.	0·165		0·56		0·41		0·034	

* Less than 0·021.

more severe the opacity, as judged by slit-lamp appearance, the greater the reduction in GSH content of the lens. In no. 18, which had no opacity, the GSH had already fallen by 13%; in nos. 19 and 20 there was no significant change. Thus the GSH content, while unaffected on the day following irradiation, began to decrease before any visible change had occurred in the lens and continued to fall as the degree of opacity increased. The GSH of the left lenses were all within normal limits (Bellows, 1944).

The PSH content of lens extract was compared in ten rabbits. In general, the decrease in PSH in the affected lens was greater the more severe the cataract, but in all cases the decrease was less than that of the GSH. Furthermore, there was no change in PSH in rabbits nos. 15, 17 and 18, whereas the GSH was significantly diminished. Thus it appears that GSH is affected before PSH and to a greater extent.

The PSH content of the suspension was reduced to a rather smaller extent than that of the extract (Table 1). As there appears to be little change in the SH content of the insoluble protein (i.e. difference between values for suspension and extract) (Table 1), the decrease in SH must be mostly in the soluble protein.

Effect of irradiation on enzymes in lens

Of the five enzymes which have been compared (Tables 1 and 2) only one, glutathione reductase, was affected by irradiation.

Glutathione-reductase. This is the only enzyme in lens known to use GSSG as its substrate and so it is interesting to find, in each of twelve comparisons, that enzyme activity was less in extracts of the right, cataractous, lens than it was in extracts of the corresponding left lens (Table 1). (The results are expressed as mg. α-ketoglutaric acid formed by the whole lens/hr. by coupling the reductase, through TPN, with the decarboxylation of *iso*citrate (see Methods section).) There was no difference in activity between the right and left lenses of the rabbits killed on the day after irradiation (nos. 19 and 20), and in comparisons between extracts of lenses from five normal rabbits the activity of the right and left lenses did not differ by more than 5%. The decrease in GSSG-reductase activity could be detected as soon as there was any sign of the opacity in the lens. In rabbits nos. 15–17 there was some decrease in both GSH and GSSG reductase, while the PSH was unchanged.

Malic enzyme. In the lenses of seven normal rabbits the difference in activity between the right and left lens of the same animal was never more than 13%. The activities ranged between 1·2 and 1·8. Table 2 shows that there was no consistent difference between the right and left lenses of four experimental animals, and the activities were of the same order as in the normal animals.

Table 2. *Comparison between right (irradiated) and left lenses. Activity of malic enzyme, isocitric and lactic dehydrogenases and cytochrome-reductase*

(The rabbits are listed in order of decreasing severity of lens opacity. The methods of test of the individual enzymes are given in the Methods section.)

		$\Delta \log I_0/I \times 10^2$/min. for the whole lens			
No.		Malic enzyme	*iso*Citric dehydrogenase	Lactic dehydrogenase	Cytochrome reductase
1	R	—	—	—	35
	L	—	—	—	39
2	R	1·2	2·8	—	—
	L	0·8	2·5	—	—
3	R	—	—	130	42
	L	—	—	131	23
7	R	—	—	220	43
	L	—	—	260	56
8	R	—	—	318	—
	L	—	—	318	—
10	R	1·7	1·3	—	—
	L	1·8	1·1	—	—
11	R	1·3	2·8	—	—
	L	1·2	2·1	—	—
12	R	1·5	2·3	—	—
	L	1·8	3·0	—	—

iso*Citric dehydrogenase.* The activity of this enzyme was compared between the right and left lenses of three normal rabbits. They differed by 3, 12 and 33% respectively, and the activities ranged from 1·8 to 2·6. A comparison of the right and left lenses of four experimental animals showed that the *iso*citric-dehydrogenase activities did not differ significantly and were of the same order as in the normal animals.

Lactic dehydrogenase and cytochrome reductase. These two enzymes are both much more active in lens extracts than are the malic enzyme or *iso*citric dehydrogenase. Each enzyme was estimated in three irradiated animals and there was no consistent difference in activity between the right and left lenses.

Effect of irradiation on ascorbic acid in the eye

The concentration of ascorbic acid in all parts of the normal rabbit eye is many times greater than that of the blood. In any particular animal the level varies with age and with intake of ascorbic acid in food but it is the same in the two eyes of one animal. Goldmann & Buschke (1935–6) found that the ascorbic acid of the aqueous humour in the two eyes of one rabbit might differ by ± 4·1 mg./100 ml., and consider that variations larger than this are abnormal.

Table 3. *Ascorbic acid content of aqueous humour and vitreous body in right (irradiated) and left eyes*

(The rabbits are listed in order of decreasing severity of lens opacity. Nos. 1–8 had opaque lenses when killed. Ascorbic acid was estimated by titration with 2:6-dichlorophenolindophenol (see Methods section).)

	Ascorbic acid (mg./100 g.)					
	Aqueous humour			Vitreous body		
No.	Right	Left	Difference	Right	Left	Difference
1	29	34	−5	20	18	+2
2	—	40	—	15	14	+1
3	24	32	−8	13	13	0
4	49	52	−3	30	17	+13
5	29	33	−4	19	14	+5
6	25	26	−1	18	14	+4
7	12	30	−18	14	13	+1
8	40	46	−6	37	25	+12
9	30	40	−10	16	14	+2
10	12	29	−17	18	12	+6
11	49	64	−15	25	19	+6
12	15	22	−7	12	12	0
13	15	20	−5	8	8	0
14	29	29	0	22	20	+2
15	34	37	−3	17	14	+3
16	32	28	+4	14	13	+1
18	35	40	−5	17	19	−2
19	49	44	+5	19	16	+3
20	44	35	+9	18	16	+2

We have estimated ascorbic acid in the aqueous humour and vitreous humour and occasionally in the lens. Table 3 shows that of sixteen irradiated rabbits with varying degrees of lens opacity, nine have lower ascorbic acid in the aqueous humour from the irradiated right eye and six, on the basis of Goldmann & Buschke's results, show no change. The two rabbits killed 20 hr. after irradiation show no change. Of those showing a reduction in ascorbic acid four had opaque lenses and five were killed before the opacity was fully developed. Of those showing no change three had complete and three incomplete opacities. It therefore seems that though there is a tendency for the ascorbic acid of the aqueous humour to fall after irradiation the fall is not correlated with opacity formation in the lens.

The ascorbic acid of the vitreous body is lower than that of the aqueous humour. After irradiation there is no decrease and in some cases there seems even to be an increase in ascorbic acid content. It may be that the level of ascorbic acid, like that of glucose (Adler, 1930), is dependent on retinal metabolism. Damage to the retina by irradiation might diminish its use of ascorbic acid which would then tend to accumulate in the vitreous body.

We have been able to estimate ascorbic acid in only a few lenses, but have found that there is little change even when the opacity is far advanced. For example, in rabbit no. 1 the right lens contained 27 mg. ascorbic acid/100 g. and the left lens 23 mg./100 g.

DISCUSSION

The course of development of cataract in these rabbits is the same as that described by Cogan & Donaldson (1951) who concluded that '...the lens is a relatively susceptible organ to irradiation with energies used in therapy (200 kV.–1000 kV.) and that the threshold for the production of cataract is less than that for any other gross abnormality of the eye or periocular tissue...'. The earliest changes found in the lens after irradiation occur in the dividing cells of the epithelium. von Sallmann (1952) found complete inhibition of mitosis 2 hr. after a dose of 1500 r. to a rabbit. This inhibition persisted for 3–4 days and was followed a few days later by excess mitoses. Nuclear fragmentation and extrusion of Feulgen-positive materials could be seen 2 hr. after irradiation.

von Sallmann (1951), using a staining reaction, found no change in deoxyribonucleic acid (DNA) of the epithelium up to 3 days after irradiation, but 2–3 weeks later the DNA was lower in the irradiated lens. von Sallmann & Locke (1951) found that lenses with complete irradiation cataract took up more radioactive sodium and iodine and less radioactive potassium and phosphorus than normal, but early cataract changes did not alter phosphorus uptake. In fact, no early biochemical change has been found after irradiation, yet it would seem possible that irradiation causes some change in all lens cells, not only in the epithelium, to lead to complete opacity and disorganization.

The earliest change we have found is diminution of GSH which begins before any abnormality is visible by slit-lamp examination of the lens. Bellows & Rosner (1937) showed a decrease of GSH in the lenses of rats fed galactose before any opacity developed, and Bellows & Schoch (1950) found that GSH diminished in rabbit lenses 72 hr. after alloxan treatment, if by then the rabbit had developed a high blood sugar; the first abnormality in the lens *in vivo* was noticed at the first weekly examination with the slit-lamp. Waters (1950), who also examined the lenses of rabbits made diabetic with alloxan, found that while the GSH remained within normal limits the protein SH was significantly lowered immediately after injection and did not regain its normal value even after 2 years. Dische & Zil (1951) found reduction in both PSH and GSH in human senile cataract.

In our experiments the first signs of clinical opacity were accompanied by a loss in GSSG-reductase activity and a further decrease in the GSH content. On the basis of this correlation between GSH and enzyme activity it is tempting to consider that glutathione reductase plays a part in the changes in SH occurring in cataract formation, especially as it is the only enzyme we have found to be affected. It is remarkable to find that in the opaque lens the activity of the other enzymes tested so far, cytochrome reductase, lactic dehydrogenase, *iso*citric dehydrogenase and malic decarboxylase is normal.

The fact that the loss in PSH began at a later stage in the development of opacity than that of GSH, and was always less pronounced, suggests that the level of PSH is maintained to some extent at the expense of GSH. Some of the protein SH will consist of SH groups in enzymes. Experiments are being done to find out whether enzymes known to contain functional SH groups are affected in the development of radiation cataract.

SUMMARY

1. Decrease of reduced glutathione occurred in the lens after X-radiation before any clinical change could be detected. Loss of reduced glutathione increased as the opacity progressed.

2. Protein SH groups decreased at a later stage of cataract formation than reduced glutathione, and to a smaller extent.

3. Decrease of reduced glutathione in lens was accompanied by a decrease of glutathione-reductase activity. The activity of four other enzymes was unaffected even in completely opaque lenses.

4. There was a slight fall of ascorbic acid in the aqueous humour after irradiation, but it was not correlated with the formation of opacity in the lens. There was no decrease of ascorbic acid in the lens or vitreous body.

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The Metabolism of ^{35}S-labelled 2-Thiolhistidine and Ergothioneine in the Rat

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The origin and function of ergothioneine in blood are unknown; its presence, however, is well established (see Heath, Rimington, Searle & Lawson, 1952, for a general review). Until the discovery by Leone & Mann (1951) of ergothioneine in the seminal vesicle secretion of the boar, the only report of ergothioneine in animals was in blood cells. Seminal ergothioneine is present in high concentration, 40 mg./100 ml. of vesicular secretion as opposed to 6 mg./100 ml. in whole blood. Although blood ergothioneine has been shown to be present in all species so far examined, seminal ergothioneine appears to be limited to one species.

Spicer, Wooley & Kessler (1951) have shown that when rabbits are reared on a controlled diet, the blood is nearly or completely devoid of ergothioneine; but when the diet is supplemented with either ergothioneine or liver extract then increased ergothioneine blood levels are observed. Heath *et al.* (1952) have also shown that dietary ergothioneine appears in the red cells of rats. The incorporation was found to be gradual, possibly occurring during erythropoiesis, and was not due to diffusion of ergothioneine from the plasma into the cells. Evidence that a diet, not containing added ergothioneine, can affect the ergothioneine content of the blood has been produced by Potter & Franke (1935) for rats and by Eagles & Vars (1928) for pigs. Spicer *et al.* (1951) have shown that the ergothioneine content of rabbit blood bears a relationship to the rate of formation and reversion of methaemoglobin after treatment of the erythrocytes with sodium nitrite.

Recent studies of the racial differences in the level of blood ergothioneine have been made by Fraser (1951) and by Touster & Yarbro (1952). Eskimos, Canadian Indians and Negroes all have higher levels than a white population. Fraser (1951) precluded the possibility of a dietary effect. Touster & Yarbro (1952) report lower blood levels in infants than adults, and are in agreement with Fraser (1951) that there is no significant variation at other ages.

Ergothioneine is the betaine of 2-thiolhistidine, and the latter compound could be its biological precursor. Thiolhistidine has never been isolated from natural material but it has been synthesized by Ashley & Harington (1930) and Harington & Overhoff (1933). Eagles & Vars (1928) suggested that maize might contain thiolhistidine as a precursor of ergothioneine, but Hunter (1951) failed to find any precursor of ergothioneine in maize. Ashley & Harington (1930) also failed to find thiolhistidine in zein. The only report of the possible natural occurrence of thiolhistidine is by Lefevre & Rangier (1942) who showed that 12% of the unidentified sulphur in horse serum proteins was precipitated with mercuric chloride and oxidized to sulphate with bromine water, both properties being characteristic of 2-thiolhistidine.

The synthesis of ergothioneine by Heath, Lawson & Rimington (1951) has made possible the preparation of isotopically labelled ergothioneine. This has now been used to study further the effect of feeding ergothioneine to rats. ^{35}S-labelled 2-thiolhistidine has also been prepared to see whether this compound can be methylated *in vivo* to yield ergothioneine, or whether it can be metabolized in any other way by the rat.

MATERIALS AND METHODS

[^{35}S]2-*Thiolhistidine and ergothioneine.* These were synthesized by the method of Heath *et al.* (1951) using 25 g. $KCN^{35}S$ containing 10 mc. ^{35}S, obtained from the Radiochemical Centre, Amersham.

Perchloric acid mixture. 1 vol. 60% $HClO_4$ + 3 vol. 70% HNO_3.

'*Carrier*' H_2SO_4. 0·01714 N-H_2SO_4, standardized gravimetrically so that 25 ml. yielded 50 mg. $BaSO_4$.

Animals. Albino rats (100 g.) were used. These were fed a standard pellet diet (diet 41, Associated London Flour Millers Ltd., Bankside House, London, E.C. 3) and tap water, *ad lib.*, and weighed daily.

Administration of (*a*) [^{35}S]2-*thiolhistidine and* (*b*) [^{35}S]-*ergothioneine.* (*a*) Three male rats were allowed a 40 mg./100 ml. solution of [^{35}S]thiolhistidine in tap water *ad lib.* for 21 days (the total weight of thiolhistidine ingested was 400 mg. containing 200 μc. [^{35}S]). (*b*) Two male rats and one female were allowed a 25 mg./100 ml. solution of [^{35}S]ergothioneine in tap water *ad lib.* for 21 days (the total weight of ergothioneine ingested was 250 mg. containing 125 μc. [^{35}S]).

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Control rats were kept in all experiments. Urine and faeces were collected daily, and at the end of 21 days all rats were given tap water *ad lib.* in place of the radioactive solutions. The urine was examined daily for radioactivity and when this had reached a constant low level the rats were chloroformed and blood immediately removed from the heart and treated with heparin.

Determination of ergothioneine in blood. Ergothioneine was determined by the method of Hunter (1949) incorporating the modification of Fraser (1951). Whole blood (2 ml.) was used and the value per 100 ml. corpuscles calculated from the haematocrit.

Determination of ergothioneine in urine and tissues. Ergothioneine was determined as described by Heath *et al.* (1952), except that the colour intensities were determined at a wavelength of 525 mμ. with a Unicam diffraction grating spectrophotometer.

Determination of ^{35}S *distribution in tissues.* Except in the cases of blood, bone marrow and hair, either the whole organ (if the wet weight was less than 1 g.) or 1 g. of a representative sample of larger organs was rinsed with 0·9% (w/v) NaCl to remove surface blood, prior to wet oxidation. *Blood.* The total volume of blood (3–5 ml.) remaining after ergothioneine and haematocrit determinations was separated into cells and plasma. The cells were washed seven times with 5 ml. portions of 0·9% (w/v) NaCl, the first washing being added to the plasma fraction. *Bone marrow.* After removal of all tissue from the limbs, the bones were crushed and extracted with 15 ml. water. *Hair.* The hair was shaved from the back and washed thoroughly with hot soap solution; 0·3 g. dried hair was used.

The material prepared as described above was refluxed for 18 hr. with 15 ml. perchloric acid mixture and 0·1 g. Cu wire in 50 ml. Kjeldahl flasks. The solutions were evaporated, the residues dissolved in 4 ml. 6N-HCl and again evaporated to dryness, redissolved in 15 ml. boiling water with the addition (if necessary to effect complete solution) of 0·1 ml. 6N-HCl. 'Carrier' H_2SO_4 (10 ml.) was added to the solutions thus obtained from the heart, lung, pancreas, kidney, spleen, eyes, tongue, brain, testis, bone marrow and seminal vesicle. The total sulphate ions were then precipitated as $BaSO_4$, collected and washed in tared 10 ml. centrifuge tubes and, after drying at 105° and reweighing, samples were transferred with 50% ethanol to tared nickel planchets. The activity was determined with an end-window counter at infinite thickness.

Fractionation of urinary sulphur. The radioactivity of the urine was too great to determine without dilution; the following method was adopted in all cases. *Free sulphate.* 1 ml. urine was mixed with 25 ml. 'carrier' H_2SO_4, 10 ml. 3N-HCl and 10 ml. 5% (w/v) $BaCl_2$. The precipitated $BaSO_4$ was collected as described above. *Ethereal sulphate.* The supernatants and washings from the free sulphate determinations were filtered through Whatman no. 42 papers, 25 ml. 'carrier' H_2SO_4 added to each and the mixture boiled for 30 min. The $BaSO_4$ samples were collected in tared 10 ml. centrifuge tubes, washed successively with water, 2N-NaOH and water until neutral. The $BaSO_4$ was dried and transferred to planchettes as described above. *Bromine-oxidizable sulphur.* The supernatant liquids and washings (prior to the NaOH wash) were filtered through Whatman no. 42 papers, 25 ml. 'carrier' H_2SO_4 and 2 ml. Br were added to each and after 30 min. excess Br was removed by boiling. The $BaSO_4$ was collected as for ethereal sulphates. *Total sulphur.* Urine (1 ml.) was oxidized with perchloric acid mixture as described above, 25 ml. 'carrier' H_2SO_4 were added and $BaSO_4$ collected as for 'free sulphate'.

Fractionation of artificial mixtures. The following solutions were fractionated as described above: (*a*) 1 ml. $H_2{}^{35}SO_4$ (a suitable dilution of a solution obtained from the Atomic Energy Research Establishment, Harwell) + 45 mg. inactive thiolhistidine, (*b*) 1 ml. [^{35}S]thiolhistidine solution (as administered to the rats), (*c*) 1 ml. [^{35}S]ergothioneine solution (as administered to the rats).

Fractionation of sulphur in liver and kidney from rats fed with [^{35}S]*ergothioneine.* The bulked residues (20 g.) of 3 livers after ergothioneine and total S determinations were ground and suspended in water (100 ml.) in a homogenizer and mixed with ethanol (100 ml.). After centrifugation, the clear supernatant solution was evaporated to dryness under reduced pressure. The residue was extracted with 2 ml. 2·3% (w/v) sulphosalicylic acid, filtered and made up to 3 ml. with water. 1 ml. of this solution was fractionated as described above for urine. The ethanol-precipitated protein was washed with 50% (v/v) ethanol, dried, and 0·5 g. oxidized with perchloric acid mixture. The $BaSO_4$ was isolated as described above but without the addition of carrier. The bulked residues (1 g.) of the kidneys of the three ergothioneine-fed rats were treated in the same way.

Paper chromatography. Extracts from the livers of normal, ergothioneine and thiolhistidine fed rats, made as described above were fractionated by single-dimension paper chromatography using butanol/acetic acid/water mixtures (50:10:40). Owing to the high concentration of soluble material other than ergothioneine the identification of the ergothioneine on the chromatograms was made by adding 30 μg. of ergothioneine to an equal portion of each extract and using this solution as a reference marker. The position of the ergothioneine after development of the chromatograms was found by spraying with either a 0·4% (w/v) ethanolic solution of 2:6-dichloroquinone chloroimide (British Drug Houses Ltd.) or by an equal mixture of the buffer and diazo solutions described by Hunter (1951), followed by 2N-NaOH.

RESULTS

As one object was to ascertain whether the sulphur of 2-thiolhistidine was utilized by the rat, it was essential to be certain that circulating thiolhistidine, which was in process of elimination, was not confused with any thiolhistidine permanently incorporated into the system.

Distribution of ^{35}S *in urine*

As can be seen from Table 1, 4 days after discontinuing the administration, the radioactivity in all fractions of the urine was negligible; the first thiolhistidine rat was killed on the 29th day of the experiment.

The bromine-oxidizable sulphur fraction, which represents the unchanged mercaptoglyoxaline nucleus of thiolhistidine accounts on the 21st day for approximately 90% of the total radioactivity and the free sulphates and ethereal sulphates, which are some indication of the degree of metabolism, for 5 and 1% respectively.

A similar experiment on the urinary excretion of [^{35}S]ergothioneine was also carried out.

Table 1. *Radioactivity of* $BaSO_4$ *(counts/min. in 1 sq.cm. samples of infinite thickness) prepared by fractionation of the sulphur-containing constituents of the urine obtained from rats maintained on a diet containing* [^{35}S]*thiolhistidine*

Days from commencement of feeding	Total S	Free sulphate	Ethereal sulphate	Bromine-oxidizable S
1–20 bulked	747	44	10	911
21	788	50	13	959
22*	401	50	15	420
23	53	10	1	43
24	13	0	4	13
25	4	0	1	2
26	3	0	0	5

* [^{35}S]Thiolhistidine was fed continuously for 21 days and discontinued from the 22nd day onwards. No ergothioneine could be detected colorimetrically in the urine.

Table 2. *Radioactivity of* $BaSO_4$ *(counts/min. in 1 sq.cm. samples of infinite thickness) prepared by fractionation of the sulphur-containing constituents of the urine obtained from rats maintained on a diet containing* [^{35}S]*ergothioneine*

Days from commencement of feeding	Total S	Free sulphate	Ethereal sulphate	Bromine-oxidizable S
1–20 bulked	180	63	9	136
21	211	71	10	169
22*	97	28	1	104
23	19	3	1	16
24	18	5	2	16
25	12	5	6	10
26	14	1	3	8

* [^{35}S]Ergothioneine was fed continuously for 21 days and discontinued from the 22nd day onwards. The bulked urine (days 1–20) contained 12 mg. ergothioneine/100 ml.

As shown in Table 2 the radioactivity fell considerably when the administration was discontinued, although not to such an extent as in the thiolhistidine experiment. The bromine-oxidizable sulphur fraction accounts for 60% of the total radioactivity and the free and ethereal sulphates for 35 and 4% respectively.

In order to prove that this method of sulphur fractionation was valid and coprecipitation did not occur, mixtures of $H_2{}^{35}SO_4$ and thiolhistidine, H_2SO_4 and [^{35}S]thiolhistidine, and also of H_2SO_4 and [^{35}S]ergothioneine were fractionated by the same method. In all cases good separation was obtained (Table 3). The $BaSO_4$ derived from the free sulphate

Table 3. *Radioactivity of* $BaSO_4$ *(counts/min. in 1 sq.cm. samples of infinite thickness) prepared from mixtures of active and non-active ergothioneine, thiolhistidine and* H_2SO_4

Constituents of mixture	Free sulphate	Ethereal sulphate	Bromine-oxidizable S
[^{35}S]Thiolhistidine + H_2SO_4	0	0	541
[^{35}S]Ergothioneine + H_2SO_4	0	0	182
Thiolhistidine + $H_2{}^{35}SO_4$	428	0	0

was not contaminated with any [^{35}S]thiolhistidine or [^{35}S]ergothioneine, nor was either of these compounds oxidized to sulphate, during the procedure for hydrolysis of ethereal sulphate.

Ergothioneine in tissues

The results of the colorimetric determinations of ergothioneine in blood and tissues are shown in Table 4.

All rats were fed for 21 days with either ergothioneine or thiolhistidine. Owing to practical limitations only one rat was killed at a time and complete analyses were carried out. As shown in Table 4 the feeding of thiolhistidine does not cause any increase in blood ergothioneine level, but ergothioneine is present in the liver and kidney.

Table 4. *Ergothioneine distribution in rats maintained for 21 days on a diet containing either ergothioneine or thiolhistidine*

Diet	Time after end of administration (days)	Blood cells (mg./100 ml.)	Liver (mg./100 g.)	Kidney (mg./100 g.)	Spleen (mg./100 g.)
Control 1	—	1·8	3·0	2·8	<1·0
Control 2	—	1·4	3·8	3·0	<1·0
Control 3	—	1·8	3·8	3·0	<1·0
Thiolhistidine 1	7	2·1	10·5	8·0	<1·0
Thiolhistidine 2	10	1·7	10·5	7·8	<1·0
Thiolhistidine 3	26	1·2	8·0	4·1	<1·0
Ergothioneine 1	10	21·1	31·5	21·0	2·0
Ergothioneine 2*	16	12·8	16·0	12·2	1·2
Ergothioneine 3	19	15·4	26·0	14·0	1·4

* Ergothioneine-fed rat no. 2 was pregnant at the time of death.

Administered ergothioneine causes an increase in the blood level from a mean value of 1·7 mg./100 ml. to as high a value as 21·1 mg./100 ml. The increase in the liver and kidney is also much more pronounced than in the case of the thiolhistidine experiment.

No ergothioneine was found in the seminal vesicles or testes of any of the rats. The placenta of the pregnant female ergothioneine-fed rat contained 6 mg./100 g. of ergothioneine, but no ergothioneine could be detected in the foetuses.

The distribution of ^{35}S in the rats after administration of [^{35}S]thiolhistidine and [^{35}S]ergothioneine is given in Table 5.

The very low activities recorded for the rats fed [^{35}S]thiolhistidine show conclusively that the sulphur of thiolhistidine is not permanently utilized in the metabolism of the rat.

In the ergothioneine experiment some radioactivity was found in all organs. There was high activity in the blood cells, and although surface blood was removed by washing with 0·9% (w/v) sodium chloride prior to oxidation, sufficient blood probably remained in the tissue to account for the radioactivity found in the eyes, tongue, muscle, pancreas, brain, foetus, placenta, testis and epididymis, seminal vesicle and spleen. Radioactivity of the barium sulphate obtained from the heart and lungs was too great to be accounted for in this manner (ergothioneine, however, was not estimated colorimetrically in the heart and lungs). Besides incorporation in the blood cells (compare Table 4) ergothioneine is without doubt accumulated by the bone marrow, liver and kidney.

The radioactivity due to the ergothioneine present in the liver, kidney and blood cells of the [^{35}S]ergo-

Table 5. *Radioactivity of* $BaSO_4$ *(counts/min. in 1 sq.cm. samples of infinite thickness) prepared from the total sulphur of tissues obtained from rats maintained for 21 days on a diet containing* [^{35}S]*thiolhistidine or* [^{35}S]*ergothioneine*

(Values are corrected for added carrier, background and decay. The figures in brackets are the number of days after the last day of administration of ergothioneine or thiolhistidine before the rats were killed. No correction has been made for the sulphur content of the very small amount of heparin used.)

	Rats on [^{35}S]ergothioneine diet			Rats on [^{35}S]thiolhistidine diet		
Organ	Rat 1 (10)	Rat 2* (16)	Rat 3 (19)	Rat 1 (7)	Rat 2 (10)	Rat 3 (26)
Liver	671	286	480	0	2	2
Kidney	425	190	195	8	3	0
Blood cells	408	242	301	4	1	0
Bone marrow	229	134	174	0	0	1
Placenta	—	120	—	—	—	—
Lungs	177	107	122	5	0	2
Heart	90	64	71	3	0	0
Testes and epididymis	66	—	41	7	2	2
Spleen	48	91	69	0	0	0
Blood plasma	43	12	22	2	2	0
Muscle	39	79	41	2	0	4
Tongue	38	25	24	0	6	0
Foetus	—	22	—	—	—	—
Brain	26	14	25	3	0	0
Pancreas	26	24	26	0	0	0
Seminal vesicles	26	—	43	0	0	0
Eyes	17	8	9	0	0	0
Hair	5	1	4	7	1	0
Faeces	—	—	7	—	—	0

* This rat was pregnant at time of death.

Table 6. *Comparison of the relative specific radioactivities of* $BaSO_4$ *samples (counts/min. in 1 sq.cm. of infinite thickness) prepared from the total sulphur of the liver, kidney and blood cells of rats fed* [^{35}S]*ergothioneine with calculated radioactivities based on the content of ergothioneine* determined colorimetrically*

	Rat 1		Rat 2		Rat 3	
Tissue	Found	Calculated	Found	Calculated	Found	Calculated
Liver	671	670	286	250	480	500
Blood cells	408	417	242	267	301	330
Kidney	425	226	190	98	195	98

* Ergothioneine (1 mg.) after oxidation and dilution with H_2SO_4 so as to yield 100 mg. $BaSO_4$ had an activity of 494 counts/min./sq.cm. at infinite thickness.

Table 7. *Radioactivity of* $BaSO_4$ *(counts/min. in* 1 *sq.cm. samples of infinite thickness) obtained by fractionation of the sulphur constituents of the liver and kidney of rats maintained for* 21 *days on a diet containing* [^{35}S]*ergothioneine*

(Values are corrected for background and added carrier.)

	Liver		Kidney	
Fraction	Activity	Total ^{35}S in ethanol extract (%)	Activity	Total ^{35}S in ethanol extract (%)
Total S in protein	10	—	8	—
Total S in ethanol extract	675	100	52	100
Free sulphate in ethanol extract	0	0	102	9
Ethereal sulphate in ethanol extract	0	0	0	0
Bromine-oxidizable S in ethanol extract	2925	97	132	75

thioneine-fed rats was calculated from the relative specific activity of the ergothioneine administered and the amount of ergothioneine present, determined colorimetrically.

As can be seen from Table 6, the ergothioneine present in the liver and blood cells accounts for all the radioactivity, but the ergothioneine present in the kidney accounts for only 50 % of the activity. Confirmation of this was obtained by fractionation of the sulphur constituents of the aqueous 'homogenates' of the bulked residues of liver and kidney obtained from the three ergothioneine-fed rats. The results of these fractionations are shown in Table 7.

The radioactivity in the liver is therefore concentrated in the bromine-oxidizable sulphur fraction, whereas only 75 % of the activity in the kidney is in the same fraction. The general conclusion which can be drawn from all the data concerning the ergothioneine-fed rats is that dietary ergothioneine without doubt accumulates in certain tissues, but its sulphur does not appear to be utilized for the synthesis of other sulphur-containing constituents.

In the various experiments no difference in growth rate was observed between experimental animals and controls.

DISCUSSION

The only suggested biological function of 2-thiolhistidine has been as a precursor of ergothioneine or of histidine (Eagles & Vars, 1928; Neuberger & Webster, 1946). The results recorded in this paper demonstrate that thiolhistidine is not a precursor of ergothioneine, at least in the case of the rat; after 21 days continuous administration of [^{35}S]thiolhistidine there was no increase in the blood ergothioneine level. That the thiolhistidine had been absorbed from the alimentary tract was clearly shown by the urine analyses; but the complete absence of any radioactivity in all the organs of the rats examined showed that the thiolhistidine was neither accumulated as such nor was the sulphur it contained used for the biosynthesis of other compounds. This result was not entirely unexpected, since thiolhistidine has never been shown to occur naturally even in the numerous studies of amino-acids in natural materials made during recent years by the sensitive method of paper chromatography.

The appearance of non-radioactive ergothioneine in liver after feeding [^{35}S]thiolhistidine was most unexpected. Had the ergothioneine found in the liver been derived directly from the administered thiolhistidine, the relative specific activity of the barium sulphate isolated, would have been approximately 200. The possibility that the radioactivity of the administered thiolhistidine was too low for the detection of activity in the liver can therefore be excluded. The evidence for the presence of ergothioneine depends upon the highly specific Hunter colour reaction. In all cases a characteristic magenta colour was obtained. The specificity of the Hunter reaction is discussed by Lawson, Morley & Woolf (1951), and the only other compound found by these workers to give a positive Hunter reaction which could have been confused with that of ergothioneine was 2-mercaptourocanic acid which is formed from ergothioneine by the loss of trimethylamine. The possibility that the positive reactions of the liver extracts were given by 2-mercaptourocanic acid can be ruled out, because this compound is not precipitated by the iodobismuthous acid used in the analytical procedure. Supporting evidence for the presence of ergothioneine in the liver extracts was given by paper chromatography.

No attempt is made here to account for the presence of inactive ergothioneine in the livers of rats fed with radioactive 2-thiolhistidine. However, one can at least tentatively suggest that the thiolhistidine activates a reaction mechanism involving the biosynthesis of ergothioneine. Some indication that thiolhistidine may have been involved in a metabolic reaction is given by the results of the urine analyses. Although 90 % of the thiolhistidine was excreted unchanged, 5 % of the sulphur was oxidized to sulphate.

In contrast to thiolhistidine, ergothioneine is absorbed and retained by the rat. This confirms the work of Heath *et al.* (1952) and Spicer *et al.* (1951) who fed non-isotopic ergothioneine to rats and

rabbits respectively and obtained marked increases in blood ergothioneine levels.

Whether blood ergothioneine is endogenous or exogenous in origin is unknown. The fact that dietary ergothioneine is incorporated into the blood does not prove that blood ergothioneine is, in actual fact, of exogenous origin.

The hypothesis of Heath *et al.* (1952) that ergothioneine is incorporated into the blood during erythropoiesis is supported by the presence of radioactive ergothioneine in the bone marrow. Lutwak-Mann (1952) has recently reported the presence of ergothioneine in the bone marrow of normal rats. The incorporation of ergothioneine into erythrocytes is not of a transient nature. This is shown by the abnormally high blood levels recorded 19 days after the administration of ergothioneine was stopped. The results of these experiments also confirm previous reports based on colorimetric evidence (Hunter, 1949), that blood ergothioneine is confined to the cellular fraction. The small amount of ^{35}S present in the plasma was probably due to haemolysis.

The non-appearance of ergothioneine in the seminal vesicle is interesting in view of the results of Leone & Mann (1951), who showed that ergothioneine was present in boar but absent from rat seminal-vesicular secretion. Thus it appears that if ergothioneine is not a normal constituent of the seminal vesicle of any particular species, then dietary ergothioneine will not be incorporated into this organ, and the non-occurrence of ergothioneine in these organs of normal rats cannot be due to a deficiency of ergothioneine in the diet.

In view of the interest shown in recent years in the effect of ergothioneine on the thyroid, it is interesting to note the non-accumulation of ergothioneine by the thyroid gland.

There is no evidence that the sulphur of ergothioneine is used in the biosynthesis of other compounds; all the radioactive sulphur of the liver can be accounted for by the ergothioneine present. On the other hand, 35 % of the urinary ^{35}S is in the form of sulphate, and the possibility that the residue of the ergothioneine molecule is used in biosynthesis must await ^{14}C experiments. The significance of ergothioneine accumulation in the liver is as yet unknown. No explanation of its function, based on these ^{35}S studies, can be put forward. A fuller understanding of the metabolic function of this compound will probably be derived from similar studies with ergothioneine labelled in the methyl groups.

SUMMARY

1. [^{35}S]2-Thiolhistidine and [^{35}S]ergothioneine were synthesized, and administered separately, in the diet, to rats for 21 days. The distribution of ^{35}S and ergothioneine in the animals was determined after sufficient time had been allowed for the non-incorporated circulating material to be excreted.

2. The rate and pattern of urinary excretion was studied in both cases; 5 % of the thiolhistidine and 35 % of the ergothioneine sulphur was excreted as sulphate.

3. The sulphur of thiolhistidine and ergothioneine was not incorporated into other sulphur compounds of the rat.

4. There was no increase in the ergothioneine blood level after thiolhistidine administration, nor was there any accumulation of thiolhistidine in any tissue of the rat. It was concluded that thiolhistidine cannot be methylated by the rat to yield ergothioneine.

5. Dietary ergothioneine was incorporated into the blood cells, bone marrow, liver and kidney.

6. The presence of non-radioactive ergothioneine in the liver of rats after the administration of [^{35}S]-thiolhistidine was observed.

7. The absence of ergothioneine from the seminal-vesicular secretion of the rat (as opposed to the pig) is a true species difference and is not due to any deficiency of ergothioneine.

It is a pleasure for the author to acknowledge the technical assistance of Miss B. Tooth, and the interest and advice of Prof. C. Rimington.

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Two Selective Inhibitors of Cholinesterase

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One of the means used to characterize cholinesterases is to study their behaviour towards inhibitors. A number of substances have been used as so-called 'selective inhibitors', i.e. inhibitors which will attack preferentially either true or pseudo cholinesterase (pseudo ChE) (Adams & Thompson, 1948; Hawkins & Mendel, 1949), but in general the selectivity is not high. The most promising of the selective inhibitors appears to be di*iso*propyl phosphorofluoridate (DFP) (Hawkins & Mendel, 1947)) to which pseudo ChE appears to be about 100–200 times as sensitive as true ChE, and the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250) (Hawkins & Mendel, 1949) which selectively inhibits true ChE. However, these two, as well as several others which have been described, have the disadvantage that when applied to mixtures of the two types of ChE in concentration sufficiently high to cause 90–100% inhibition of one enzyme they also cause measurable inhibition of the other. In spite of this, much valuable work has been carried out. The selectivity of an inhibitor varies considerably according to the species of animal studied. Hawkins & Mendel (1949) found that true ChE was about 1000 times more sensitive to Nu 1250 than pseudo ChE in human or rat blood, but only 20 times in the dog and 5 times in the horse. Aldridge (1953) has reported similar species differences. Such species variation might prove embarrassing when attempting to classify enzymes which are neither true nor pseudo, such as occur in the rat (Ord & Thompson, 1951), fowl (Earl & Thompson, 1952) or sheep (Davies, Risley & Rutland, 1952). Todrick (1952) has used the selective inhibitors *N*-(2-diethylaminoethyl)phenothiazine hydrochloride (Diparcol) and the diethylaminoethyl ester of 1-phenyl*cyclo*pentane carboxylic acid (Parpanit) which inhibit pseudo ChE, and the diethiodide of 1:5-di(8′-quinolyloxy)pentane (RP 3381) and the dimethiodide of 1:5-di(*o*-dimethylaminophenoxy)-pentane (RP 3565) which inhibit true ChE. The selectivities appear to be of the order of 200–500 as measured by the ratio I_{50} (rat brain ChE):I_{50} (rat intestinal mucosa ChE), but comparison with other selective inhibitors is difficult, partly because the ChE of rat intestinal mucosa is not quite the same as the classical pseudo ChE (Ord & Thompson, 1951), and partly because no data are given as to the inhibition of one type of ChE when the other is about 95% inhibited. (I_{50} is the concentration giving 50% inhibition.)

It was, therefore, with great interest that we received reports of two inhibitors which appeared to have a higher selectivity than any so far studied. A personal communication from Dr W. N. Aldridge, which has since been published (Aldridge, 1953), mentioned that the pseudo ChE of horse serum was 10 000 times more sensitive than the true ChE of horse erythrocytes to *NN′*-di*iso*propylphosphorodiamidic anhydride (DPDA);* and Fulton (1952) reported that rat-brain ChE, which is predominantly true, was 50% inhibited by 10^{-6}M-dimethobromide of 1:5-di(*p*-*N*-allyl-*N*-methylaminophenyl)-pentan-3-one (284C51), whereas the hydrolysis of benzoylcholine by horse serum was unaffected by a concentration of 10^{-3}M. We have therefore examined these inhibitors more fully, in an endeavour to decide on their usefulness as tools for the study of cholinesterases. Some of the results have been the subject of a preliminary communication (Austin & Berry, 1953).

EXPERIMENTAL

Enzyme preparations. Oxalated blood was centrifuged and the erythrocytes washed twice with 0·9% NaCl. Plasma and cells were diluted as required with 0·025M-$NaHCO_3$ in order to give an output of about 200 μl. CO_2/hr. in the Warburg apparatus.

Substrates. Acetylcholine (ACh; 0·006M and 0·06M), benzoylcholine (BCh; 0·03M), acetyl-β-methylcholine (β-acetoxypropyltrimethylammonium chloride; MCh; 0·03M) and butyrylcholine (BuCh; 0·03M), were used as stock solutions in distilled water, kept in the refrigerator, and made up freshly once a week. Some data obtained by Miss J. E. Risley are reported in which propionylcholine (PrCh; 0·06M) was used. These concentrations were final.

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* This compound, $[(C_2H_7NH)_2PO]_2O$, was formerly called bis(di-*iso*propylamino)phosphonous anhydride and given the abbreviation *iso*-OMPA (Aldridge, 1953; Austin & Berry, 1953). This abbreviation incorrectly suggests that the compound is an isomer of OMPA ($[(Me_2N)_2PO]_2O$; DuBois, Doull & Coon, 1950); we therefore suggest that the use of *iso*-OMPA be abandoned in favour of the abbreviation DPDA.

Measurement of enzyme activity. The enzyme activity was measured in the Warburg apparatus and is given as the q_{CO_2} XCh, i.e. the vol. of CO_2 in μl./hr./ml. plasma or packed erythrocytes when using XCh as substrate.

For several experiments double-side-arm flasks were used, so that inhibitor and substrate could be added separately to the enzyme in the main vessel. In most experiments with 284C51 the enzyme was placed in the side arm and tipped into a mixture of substrate and inhibitor, in order to obtain equilibrium as rapidly as possible.

Inhibitors. DPDA was prepared in this Establishment. 284C51 was presented by Dr Copp of the Wellcome Research Laboratories. The inhibitors were dissolved in distilled water, final concentrations being arrived at by v/v serial dilution.

RESULTS

The first experiments were carried out with the ChE's of human blood. The plasma contains a typical pseudo ChE and the erythrocytes a typical true ChE (Adams, 1949; Adams & Whittaker, 1949).

Reversibility of the inhibition

DPDA. A dilution of human plasma in bicarbonate-Ringer (Krebs & Henseleit, 1932) was divided into two parts, one of which was treated overnight at room temperature with 5×10^{-6}M-DPDA. The activity of a sample of both portions was measured. 10 ml. of each portion were dialysed for 20 hr. at 0° against five changes of 500 ml. bicarbonate-Ringer, and the activity determined. It was presumed that dialysis removed surplus DPDA, but the data of Table 1 show that it did not cause dissociation of the enzyme-inhibitor complex.

Table 1. *Reversibility of inhibition of human plasma cholinesterase by DPDA*

	Activity (%)	
	Before dialysis	After dialysis
Control	100	95
Treated with 5×10^{-6}M-DPDA	3	4

284C51. The human erythrocyte preparation is a suspension of particles, and so lends itself to the process of washing by centrifuge, which is much less tedious than dialysis. A suspension of erythrocytes in bicarbonate-Ringer was divided into two parts, one of which contained 10^{-6}M-285C51. After 10 min. exposure to the agent, samples were taken for measurement of activity. The remainder of both portions was washed twice with 50 vol. bicarbonate-Ringer, allowing the mixture to stand 10 min. before centrifuging for 10 min. at 3500 rev./min. in an angle centrifuge. The packed cells were sampled after each washing. Table 2 shows that the inhibition was readily and completely reversed by this treatment.

Table 2. *Reversibility of inhibition of human erythrocyte cholinesterase by 284C51*

	Activity (%)
Control, before washing	100
Control, after washing twice	93
Inhibited by 10^{-6}M-284C51	7
Inhibited, washed once	72
Inhibited, washed twice	93

Combination of the inhibitors with the active centre

A competitive inhibitor may be defined as one which combines with the same active centre as the substrate. If the inhibition is reversible, the degree of inhibition varies with the concentration of both substrate and inhibitor, and a simple test for discovering this type of behaviour was given by Lineweaver & Burk (1934). This test cannot be used with true ChE assayed under the conditions here described, because the optimum ACh concentration is near the limit below which accurate measurements of enzyme activity are impossible. An alternative test is available. Augustinsson (1948) has argued that the optimum substrate concentration ought to be increased in the presence of a competitive inhibitor, and has produced some evidence in support (see also Augustinsson & Nachmansohn, 1949). It is shown in the Appendix that this assertion has a theoretical basis, and that if the inhibition is non-competitive (i.e. degree of inhibition uninfluenced by substrate concentration) the optimum is unchanged. In Fig. 1 it is shown that the optimum ACh concentration for human-erythrocyte ChE is considerably increased by 284C51, from about 0·003M to somewhere in the region of 0·1M. (The value of 0·1M is probable, although we have not

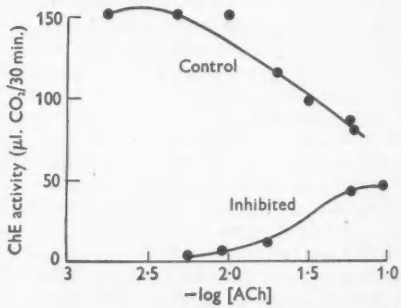

Fig. 1. Increase in the optimum ACh concentration of human erythrocyte ChE in the presence of $1{\cdot}3 \times 10^{-6}$M-284C51, showing competitive nature of the inhibition. (The increased optimum appears to be about −(log ACh) = 1.)

employed a sufficiently high ACh concentration to demonstrate that this is certainly a maximum.) It is shown in the Appendix that the optimum is increased by the factor $\left(\frac{K_i+[I]}{K_i}\right)^{\frac{1}{2}}$, so that the data of Fig. 1 suggest a value for K_i, the enzyme-inhibitor equilibrium constant, of about $1{\cdot}2 \times 10^{-9}$ M. This value is not exact because of the uncertainty of estimating the optima, but a fairly high affinity is indicated.

Neither the Lineweaver-Burk nor the Augustinsson tests can be applied to irreversible inhibitors. The reversal by substrate, if it occurs, is too slow to be measured under the conditions of such a test,

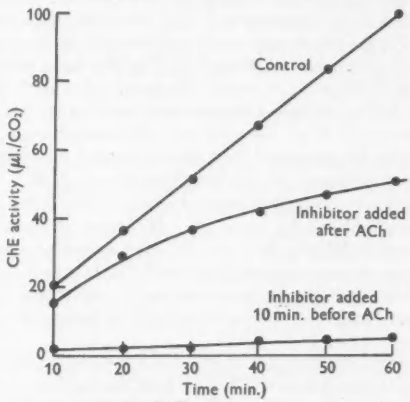

Fig. 2. Protection by ACh of human plasma ChE against inhibition by 10^{-4} M-DPDA, showing competitive nature of the inhibition.

and consequently irreversible inhibitors are apparently non-competitive (see Mackworth & Webb, 1948; Augustinsson & Nachmansohn, 1949). We have here applied the test of protection by substrate. DPDA (10^{-4} M) causes 93% inhibition of human-plasma ChE in 10 min. in the absence of ACh (Fig. 2). If the same concentration is added after ACh, the rate of inhibition is slowed down, so that the 93% level is reached only after some 60–70 min. The fact that the substrate can protect is evidence that the inhibition is competitive.

The rate of inhibition of human plasma ChE by DPDA was found to be first order for most of several concentrations of inhibitor, and was presumed so for the concentration $2{\cdot}7 \times 10^{-8}$ M (Fig. 3). The time to 50% inhibition was found inversely proportional to the concentration of inhibitor (Fig. 4). Consequently the inhibition reaction is bimolecular, with a rate constant of $4{\cdot}2 \times 10^{4}$ l. mol.$^{-1}$min.$^{-1}$.

Selectivity of the inhibitors

It seemed from the foregoing results on human blood that the selectivity of DPDA for the pseudo

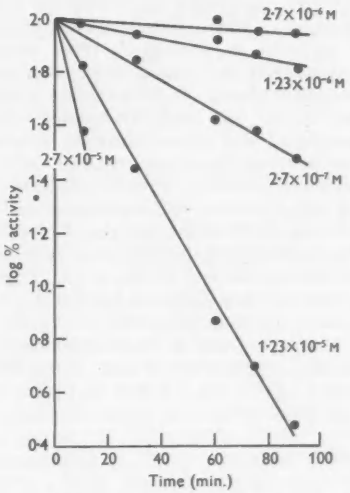

Fig. 3. Rate of inactivation of human plasma ChE by DPDA. The concentration of inhibitor is shown against each line. (The rate for $2{\cdot}7 \times 10^{-6}$ M is presumed first order.)

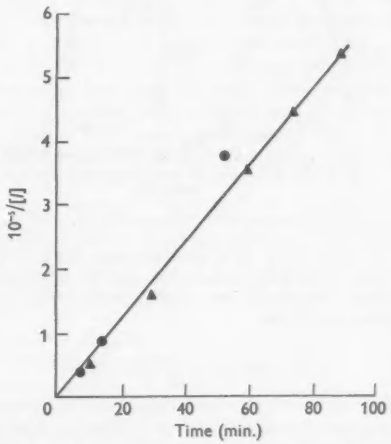

Fig. 4. Inverse relationship between concentration of DPDA and time required for 50% inhibition of human plasma ChE, showing that the inhibition reaction is bimolecular. ●, from Fig. 3; ▲, additional experiments.

ChE of the plasma was not nearly as high as had been found for horse blood. This discrepancy was the subject of correspondence between Dr Aldridge and

ourselves and was confirmed in both laboratories. We found the ratio I_{50} (cells):I_{50} (plasma) to be about 50 for human and upwards of 6500 for horse blood. Aldridge (1953) has reported 56 and 11300 respectively. According to Whittaker and his collaborators (Adams, 1949; Adams & Whittaker, 1949; Mounter & Whittaker, 1950; Sturge & Whittaker, 1950) the plasma enzymes of the two species agree closely to a substrate specificity pattern which has been designated 'butyrocholinesterase' and the erythrocyte enzymes to another 'aceto-cholinesterase' pattern. In spite of similarities in behaviour towards substrates, there seemed to be profound differences towards DPDA. Also, Fulton (1952) has claimed that 10^{-3}M-284C51 is without effect on the hydrolysis of BCh by horse serum, whereas we find an I_{50} of 3×10^{-4}M using ACh, and for human serum an I_{50} of 10^{-3}M.

We have therefore examined the species variations in the selectivity of the inhibitors, using the plasma and erythrocytes of man, rhesus monkey, horse and guinea pig, as well as plasma of the domestic fowl.

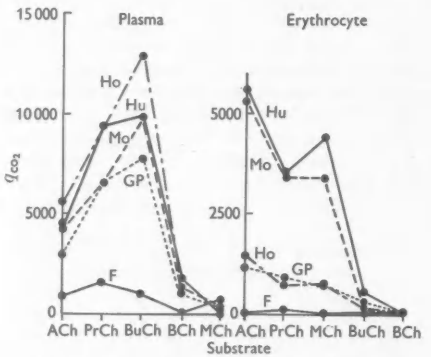

Fig. 5. Substrate specificity patterns of blood ChE's. q_{CO_2} values in µl./hr./ml. of plasma or packed erythrocytes. ACh, acetylcholine; BCh, benzoylcholine; BuCh, butyrylcholine; MCh, acetyl-β-methylcholine; PrCh, propionylcholine. Ho, horse; Hu, human; Mo, monkey; GP, guinea pig; F, fowl.

Characterization of the enzymes

The substrate specificity patterns of the enzymes in question are given in Fig. 5 (Davies, Risley & Rutland, 1952). It will be seen that the mammalian plasmas are broadly similar in the relative rates of hydrolysis of the various esters. There is a suggestion that there are two pairs of enzymes differing in the ratio $q_{PrCh}:q_{ACh}$. In human and guinea-pig plasma this is about 2, whereas in monkey and horse it is about 1·6. In all cases the $q_{BuCh}:q_{ACh}$ is about 2·3. The mammalian erythrocyte enzymes are also very similar and appear also to be divisible, on the basis of the $q_{ACh}:q_{BuCh}$ ratio, although some doubt is admissible because of the low values of q_{BuCh} and consequent inaccuracy. The enzymes of fowl blood are markedly different. Of fowl plasma more will be said later; but studies on fowl erythrocytes are profitless because of the extremely low q values.

Behaviour of the various enzymes towards the inhibitors

DPDA. Table 3 shows the concentrations of DPDA required to give 50% inhibition of the hydrolysis of various choline esters by the several plasmas. Inhibitor and enzyme were in contact for 30 min. at 38° before addition of substrate. For the mammalian plasmas the I_{50}'s for ACh, BCh and BuCh are practically identical, as might be expected if all substrates are hydrolysed by the same pseudo ChE. These I_{50}'s range between $1·3 \times 10^{-7}$ and 9×10^{-6}M: whereas for the erythrocytes the values are 3×10^{-4} to $3·6 \times 10^{-3}$M. In consequence, it might be supposed that the increased concentrations required to inhibit hydrolysis of MCh by plasma indicate that part, at least, of this hydrolysis is carried out by true ChE. However, in order to measure MCh hydrolysis with any accuracy, a very concentrated plasma was used. It is much more likely that the preparation had a relatively high binding power for inhibitor, due to substances other than ChE.

The almost equal sensitivity for all substrates in fowl plasma, and the finding that the I_{50} is much the same as with pseudo ChE, confirms the conclusion of Earl & Thompson (1952) that this tissue contains one enzyme resembling pseudo ChE and capable of hydrolysing MCh.

The selectivity of the inhibitor is fairly high, as shown by the ratios of I_{50}'s in the last column of Table 3. The shape of the percentage activity/log (inhibitor concentration) curves is such that there is a wide gap in concentration between 95% inhibition of the pseudo and 5% inhibition of true ChE. Even with human blood it appears that 3×10^{-5}M ought to give 95% inhibition of the pseudo and only 10% inhibition of true ChE. This concentration appears to be generally useful for discriminating between the two types.

284*C*51. Table 4 shows the I_{50}'s for 284C51 of the various erythrocyte preparations, using ACh and MCh. The values (ACh) range between 10^{-8} and 10^{-7}M. For pseudo ChE the values are $2·8 \times 10^{-4}$ to 10^{-2}M. The selectivity is high, at least 3000. Although the dose-response curves are flatter than with DPDA, there still exists a range of concentrations, 10^{-6} to 10^{-5}M, in which it should be possible to cause at least 95% inhibition of true but not more than 5% of pseudo ChE.

Table 3. *Inhibition by DPDA of hydrolysis of choline esters by plasma of various species. Inhibitor in contact with enzyme for 30 min. at 38° before addition of substrate*

Species	$I_{50} \times 10^7$ Acetylcholine (0·06 M)	Butyrylcholine	Benzoylcholine	Acetyl-β-methylcholine	Ratio: I_{50} (cells): I_{50} (plasma) for acetylcholine
Man	60	60	60	300	50
Monkey	1·3	1·5	1·3	1300	5000
Guinea pig	30	26	26	1600	1200
Horse	4	6	5	2500	6500
Fowl	87	87	100	87	—

Table 4. *Inhibition by 284C51 of hydrolysis of choline esters by erythrocytes of various species*

Species	$I_{50} \times 10^7$ Acetylcholine (0·006 M)	Acetyl-β-methylcholine	Ratio: I_{50} (plasma): I_{50} (cells) for acetylcholine
Man	1·0	50	10 000
Monkey	1·0	4·5	2 800
Guinea pig	0·12	0·64	217 000
Horse	1·0	1·6	2 800
Fowl plasma	$>10^5$	7 200	—

The differences in I_{50} between ACh and MCh can be rationalized when it is remembered that for a reversible competitive inhibitor the I_{50} depends on the enzyme-substrate affinity as well as on the enzyme-inhibitor affinity. In this connexion it must be observed that accurate measurement of the equilibrium constant K_s for the formation of the enzyme-substrate complex is virtually impossible using the method and conditions chosen here. We have attempted to verify this explanation for the considerable difference in I_{50}'s for fowl plasma, the more especially since this is a new type of enzyme. It is well known that $I_{50} = K_i(1 + [S]/K_s)$ whence the ratio of I_{50}'s can be calculated if the respective values of $[S]$ and K_s are known. We have found that for both ACh and MCh the relationship between activity and substrate concentration follows the Michaelis-Menten formula: the respective values of K_s are $7{\cdot}6 \times 10^{-4}$ and $1{\cdot}04 \times 10^{-2}$ M. In determining the I_{50}'s given in Table 4 the values of $[S]$ were, respectively, 6×10^{-2} and 3×10^{-2} M. The calculated ratio of I_{50}'s is therefore 20·8; found, >14. K_i is $1{\cdot}85 \times 10^{-4}$ M.

DISCUSSION

This work was undertaken to examine the value of the inhibitors DPDA and 284C51 as tools for classifying cholinesterases. Both seem to be valuable for different purposes.

If an inhibitor is to be used with a variety of substrates it should be irreversible so that variations in enzyme-substrate affinity will not affect its behaviour. DPDA appears to be very well suited to such studies, especially since our data suggest that it may be rather better than most at inhibiting pseudo ChE without affecting true ChE. This feature is not always present, to judge from results given by Hawkins & Mendel (1947, 1949) and Adams & Thompson (1948). Todrick (1952) has not yet reported on this aspect of the selective inhibitors used by him. Treatment with 3×10^{-5} M DPDA for 30 min. at 38° before addition of substrate might prove a useful means of discriminating between the two types of ChE.

If only one substrate, e.g. ACh, is to be used, 284C51 seems to be valuable, because of the wide separation of I_{50}'s between true and pseudo ChE's and because, in spite of flatter dose-response curves, there seems still to be a tenfold range of concentration, 10^{-6} to 10^{-5} M, over which it might be possible to suppress true ChE activity without there being any measurable effect on the pseudo enzyme.

It was suggested above that the plasma ChE's might be divisible into two pairs on the basis of the relative rates of hydrolysis of PrCh and ACh. These pairs were: human and guinea pig, monkey and horse. The former pair is rather less sensitive to DPDA, the I_{50}'s being $3\text{–}6 \times 10^{-6}$ M as against $1\text{–}5 \times 10^{-7}$ M (Table 3). The data are scanty and further work is necessary, but they support other findings (Hawkins & Mendel, 1949; Berry, 1951; Myers, 1952; Aldridge, 1953) that there are species differences in sensitivity to inhibitors among groups of ChE's which have much slighter differences in relative substrate hydrolysis rates. Since these phenomena have been studied in extremely crude preparations, it is not impossible that they are associated rather with variations in the non-cholinesterase fractions, competing for inhibitor, than with real differences in ChE's. When the substrate specificity pattern is markedly different,

as with fowl plasma, the tissues of rat (Ord & Thompson, 1951), sheep (Davies *et al.* 1952) or rabbit serum (Koelle, 1953) there can be less doubt that the cholinesterases themselves are different.

SUMMARY

1. Two new inhibitors of cholinesterase have been studied, namely, *NN'*-di*iso*propylphosphorodiamidic anhydride ($[(C_3H_7NH)_2PO]_2O$; bis(di*iso*propylamino)phosphonous anhydride; DPDA) and the dimethobromide of 1:5-di(*p*-*N*-allyl-*N*-methylaminophenyl)pentan-3-one (284C51). DPDA is a competitive irreversible inhibitor, selectively inhibiting pseudo cholinesterases, while 284C51 is a competitive reversible inhibitor, selectively inhibiting true cholinesterases.

2. The selectivity of the inhibitors, as expressed by the ratio I_{50} (true):I_{50} (pseudo) is generally high, although this ratio varies considerably among some mammalian species if blood erythrocytes and plasma are the sources of true and pseudo cholinesterases respectively. It appears that 3×10^{-5}M-DPDA (in contact 30 min. at 38° before addition of substrate) or 10^{-6} to 10^{-5}M-284C51 could be used to inhibit pseudo or true cholinesterase respectively without measurable effect on the other.

3. Some evidence is given suggesting that the apparently typical pseudo cholinesterases of mammalian plasmas may be capable of subclassification.

4. Further properties of fowl plasma cholinesterase are described, confirming that it is a pseudo cholinesterase in its behaviour towards inhibitors, although the substrate specificity pattern is unlike that of classical pseudo cholinesterase.

We wish to thank Dr A. H. Ford-Moore for supplies of DPDA and Dr Copp of the Wellcome Research Laboratories for the sample of 284C51. Thanks are due also to Dr W. N. Aldridge for certain of his then unpublished data.

Acknowledgement is also made to the Chief Scientist, Ministry of Supply, and to the Senior Representative, Australian Department of Supply, for permission to publish.

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APPENDIX

A Note on the Reaction with Inhibitors of Enzymes which are Inhibited by Excess Substrate

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Equations connected with enzyme kinetics are often transformed into linear form, so that experimental data may be examined with the minimum of mathematical labour (cf. Lineweaver & Burk, 1934). Transformations are used not only to determine kinetic constants, but also to establish, among other things, mechanisms of inhibition.

The equations for enzymes which are inhibited by excess substrate cannot be transformed in this manner, but give rise to quadratic or higher order curves. Consequently for some purposes, such as are discussed in the accompanying paper (Austin & Berry, 1953), tests based on linear transformations cannot be used, unless it is possible to work below

the optimum substrate concentration, where linear relationships are a good first approximation. This note mentions some deductions from the higher-order equations which do not appear to have been published.

Lineweaver & Burk (1934) have recapitulated the argument by which it was shown that the relationship between velocity (v) and substrate concentration [S], derived from the simultaneous equations,

$$E+S \overset{K_s}{\rightleftharpoons} ES \text{ (active)},$$

$$ES+(n-1)\,S \overset{K_2}{\rightleftharpoons} ES_n \text{ (inactive)},$$

is

$$v=\frac{V[S]}{K_s+[S]+\dfrac{[S]^n}{K_2}}. \qquad (1)$$

(V = hypothetical maximum velocity if ES_n were not formed.) The optimum substrate concentration is found when $dv/d[S]=0$. By differentiating equation 1 it is found that

$$[S]_{opt.}=\left(\frac{K_sK_2}{n-1}\right)^{1/n}. \qquad (1a)$$

For non-competitive inhibition the simplest treatment involves considering only the equilibrium

$$E+I \overset{K_i}{\rightleftharpoons} EI \text{ (inactive)},$$

whence the velocity in the presence of inhibitor (v') is

$$v'=v\,\frac{K_i}{K_i+[I]}. \qquad (2)$$

It is clear that $dv'/d[S]=0$ at the same value of [S] that makes $dv/d[S]=0$; i.e. in the presence of a non-competitive inhibitor the optimum substrate concentration is unaltered.

For competitive inhibition there are three simultaneous equations to solve:

$$E+S \overset{K_s}{\rightleftharpoons} ES \text{ (active)},$$

$$ES+(n-1)\,S \overset{K_2}{\rightleftharpoons} ES_n \text{ (inactive)},$$

$$E+I \overset{K_i}{\rightleftharpoons} EI \text{ (inactive)}.$$

The velocity in presence of inhibitor is now

$$v'=\frac{V[S]}{K_s\left(\dfrac{K_i+[I]}{K_i}\right)+[S]+\dfrac{[S]^n}{K_2}}, \qquad (3)$$

and by differentiating as before, the optimum substrate concentration is

$$[S']_{opt.}=\left(\frac{K_sK_2}{n-1}\,\frac{K_i+[I]}{K_i}\right)^{1/n}. \qquad (3a)$$

By comparing equations ($1a$) and ($3a$) it is seen that in the presence of a competitive inhibitor the optimum substrate concentration is increased by the factor

$$\left(\frac{K_i+[I]}{K_i}\right)^{1/n}.$$

The application of these findings is discussed in the accompanying paper (Austin & Berry, 1953).

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Hydrolysis of Benzolycholine Derivatives by Cholinesterase in Serum

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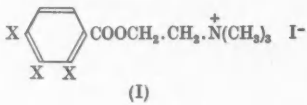
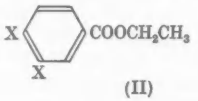
(*Received* 24 *November* 1952)

A series of derivatives of benzoylcholine (I) with substituents in ortho, meta, and para positions has been synthesized, and velocity constants of hydrolysis by horse-serum cholinesterase determined; the object of this has been to investigate the influence which the chemical reactivity of a substrate might have on its rate of reaction catalysed by an enzyme. There is already much quantitative information on changes in reactivity that can be expected from the introduction of substituents into meta and para positions in the benzene ring; the data have been summarized by Hammett (1940), who expressed these changes by the substituent constant S, where $S=\log K-\log K_0$, K being the dissociation constant of the corresponding substituted benzoic acid and K_0 the dissociation constant of unsubstituted benzoic acid. S is positive for an electrophilic substituent, negative for a nucleophilic substituent,

and zero for the unsubstituted compound. Work which is most relevant for comparison is by Tommila & Hinshelwood (1938), who studied alkaline hydrolysis of substituted derivatives of ethyl benzoate (II).



(I)



(II)

EXPERIMENTAL

Analyses were performed by Miss V. Pickerden of the National Institute for Medical Research. Melting and boiling points are uncorrected.

Materials

The method of preparing benzoylcholine iodide illustrates the general method employed for all compounds unless otherwise stated. Benzoic acid was esterified with ethylene chlorohydrin in the presence of H_2SO_4. The product distilled at 258° at atmospheric pressure and 103–108°/3 mm. Hg, and was converted by NaI in ethylmethylketone into β-iodoethyl benzoate, b.p. 135–140°/3 mm. Hg. This was dissolved at room temperature in acetone with excess trimethylamine from which the product crystallized. Recrystallized from ethanol, it had m.p. 243° (Ford-Moore, 1950).

p-*Toluoylcholine iodide*, crystallized from ethanol, had m.p. 197–198. (Found: C, 44·9; H, 5·81. $C_{13}H_{20}O_2NI$ requires C, 44·7; H, 5·73%.)

m-*Toluoylcholine iodide*, crystallized from ethanol, had m.p. 110–112°. (Found: C, 44·65; H, 5·8. $C_{13}H_{20}O_2NI$ requires C, 44·7; H, 5·7%.)

p-*Methoxybenzoylcholine iodide*, crystallized from ethanol, had m.p. 201–204°. (Found: C, 42·6; H, 5·9. $C_{13}H_{20}O_3NI$ requires C, 42·7; H, 5·5%.)

m-*Nitrobenzoylcholine iodide*, crystallized from ethanol, had m.p. 183–186° (decomp.). (Found: C, 37·95, H, 4·40. $C_{12}H_{17}O_4N_2I$ requires C, 37·9; H, 4·5%.)

p-*Fluorobenzoylcholine iodide*, crystallized from ethanol, had m.p. 173–175°. (Found: C, 40·7; H, 4·8. $C_{12}H_{17}O_2NFI$ requires C, 40·8; H, 4·8%.)

m-Fluorobenzoic acid was prepared from *m*-toluidine which was converted by the method of Balz & Schieman (1927) to *m*-fluorotoluene; this was oxidized with permanganate to *m*-fluorobenzoic acid, which was recrystallized from water, m.p. 119° (decomp.). m-*Fluorobenzoylcholine iodide*, recrystallized from ethanol, had m.p. 206–209°. (Found: C, 40·4; H, 5·2. $C_{12}H_{17}O_2NFI$ requires C, 40·8; H, 4·8%.) (Owing to shortage of material the intermediate compounds were not isolated and purified.)

m-*Chlorobenzoylcholine iodide* recrystallized from ethanol, m.p. 192–195°. (Found: C, 39·1; H, 4·5. $C_{12}H_{17}O_2NClI$ requires C, 39·0; H, 4·6%.)

o-Chlorobenzoic acid with thionyl chloride yielded *o*-chlorobenzoyl chloride. This reacted readily with ethylene chlorohydrin in the presence of pyridine. o-*Chlorobenzoylcholine iodide*, prepared from this in the usual way and recrystallized from ethanol, had m.p. 166–168°. (Found: C, 39·3; H, 4·7. $C_{12}H_{17}O_2NClI$ requires C, 39·0; H, 4·6%.)

Methods

The rates of hydrolysis were studied at 25, 30 and 37° in the usual type of Warburg apparatus. The main compartment of each manometer flask contained 2·8 ml. of 0·1 M-$NaHCO_3$ in which was dissolved 2×10^{-5} mole of the substance to undergo hydrolysis (final concentration $6{\cdot}67 \times 10^{-3}$M). CO_2 liberated from the bicarbonate solution by the free acid was measured in the manometers. The side arm contained 0·2 ml. of enzyme solution which was crude horse serum.

Experiments were performed also (at 37°) using 1×10^{-5} mole substrate and, where the reaction at this concentration was completed too rapidly for accurate determination, using 3×10^{-5} mole. As there was no significant change in rate of reaction within this range, it was assumed that the enzyme was saturated in each instance by 2×10^{-5} mole, the amount used throughout these experiments relative to 0·2 ml. of crude enzyme preparation. No determinations could be made with 5×10^{-6} mole of any of the substrates.

Two samples of horse serum were used; these were taken at different times from the same animal and gave different results in that sample 2 was more potent than sample 1. It is probable that they represent different concentrations of the same enzyme, but this assumption is in no way vital to the results reported, which are based on work with sample 2. Observations with sample 1 (see Table 1) have been repeated using sample 2.

The degree of confidence that can be placed in results is shown by the standard errors of the mean values which are included where multiple determinations were made. An approximate value only is given for the *p*-methoxy derivative as this was hydrolysed too slowly for accurate determination. Non-enzymic hydrolysis was slow compared with enzymic hydrolysis, and the errors in determination correspondingly large, and it also tended to deviate from a zero-order course. The rate of enzymic hydrolysis has therefore not been corrected for non-enzymic hydrolysis.

Further determinations were limited by the amount of the compounds available.

RESULTS

In Table 1 the zero-order velocity constants for the hydrolysis of the compounds by the two samples of horse serum enzyme are given, together with the constants for alkaline non-enzymic hydrolysis and substituent constant (S). Fig. 1 shows the correlation between reactivity of the ester linkage and rate of reaction by plotting $\log_{10} k_{37}$ against S. A linear relationship would be expected if the changes in reaction rate could be ascribed solely to the changes in reactivity caused by the substituents. Such deviations as occur can be accounted for by alterations in the 'fit' of enzyme and substrate.

Previous work, summarized by Hinshelwood (1945), has shown that the inductive effect of substituents in the benzene ring affects E, the energy of activation, and that factors concerned with molecular collision and the probability of a reaction

Table 1. *Velocity constants for substituted benzoylcholine derivatives for enzymic and non-enzymic hydrolysis and their substituent constant* S *which gives an estimate of the reactivity of the ester linkage*

(The figures in parentheses are the numbers of experiments performed. The standard error is given where more than two determinations were made.)

Derivative	Enzyme sample 1, k_{37}	Enzyme sample 2			Non-enzymic alkaline hydrolysis	
		k_{37}	k_{30}	k_{25}	k_{37}	S
m-Nitro	3·53±0·15 (4)	4·50 (1)	2·64 (1)	1·87 (1)	0·23±0·17 (3)	+0·71
m-Chloro	3·61±0·09 (4)	—	3·13 (2)	2·20 (1)	0·13±0·10 (3)	+0·37
m-Fluoro	2·39±0·03 (3)	3·12 (1)	2·70 (1)	2·39 (2)	0·22 (1)	+0·34
p-Fluoro	1·71±0·03 (4)	2·58 (1)	1·70 (1)	1·22 (2)	0·13±0·02 (3)	+0·06
Unsubstituted	1·79±0·08 (7)	2·95±0·08 (4)	1·90±0·05 (6)	1·59±0·03 (5)	0·075±0·007 (3)	0·00
m-Methyl	0·66±0·09 (4)	0·811 (2)	0·642 (1)	0·577 (1)	0·10±0·018 (3)	−0·07
p-Methyl	—	0·531 (2)	0·404 (2)	0·325 (2)	0·038 (1)	−0·17
p-Methoxy	0·1 (approx.)	—	—	—	0·065 (1)	−0·27
o-Chloro	—	2·73 (1)	1·95 (1)	—	0·189 (1)	—

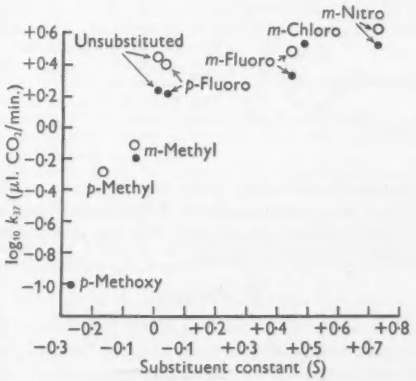


Fig. 1. Graph showing the $\log_{10}$ rates of hydrolysis, k_{37}, of the derivatives of benzoylcholine correlated with the reactivity (given by the substituent constant S). ●, Enzyme sample 1; ○, enzyme sample 2.

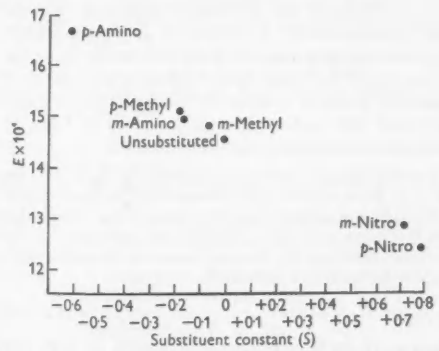


Fig. 2. Data from Tommila & Hinshelwood (1938). Correlation of energy of activation (E), with substituent constant (S) for derivatives of ethylbenzoate in alkali-catalysed hydrolysis.

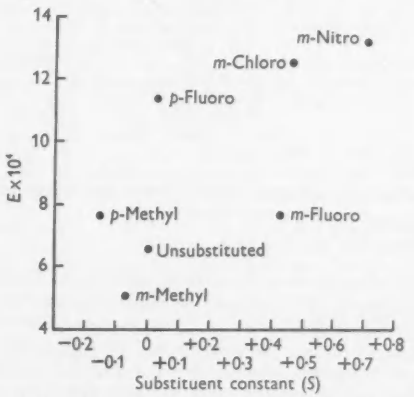


Fig. 3. Correlation of energy of activation (E) with substituent constant (S) for derivatives of benzoylcholine in enzyme-catalysed hydrolysis. The trend shown in this figure is in the opposite sense from that shown in Fig. 2.

Table 2. *Energy of activation* (E) (*affected by chemical reactivity of compounds*) *in the Arrhenius expression for hydrolysis of the ester linkage by serum cholinesterase*

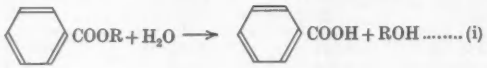
Derivative of benzoylcholine	$E \times 10^4$ (cals.)	S
m-Nitro	13·3	+0·71
m-Chloro	12·6	+0·37
m-Fluoro	7·8	+0·34
p-Fluoro	11·7	+0·06
Unsubstituted	6·3	0·00
m-Methyl	5·1	−0·07
p-Methyl	7·8	−0·17

occurring affect the non-exponential term A in the Arrhenius expression as

$$\log_{10} k = A - \frac{E}{2{\cdot}303\,RT},$$

where R is the gas constant and T the absolute temperature. The values of E were estimated by a

graphical method from the data in Table 1, and the results are shown in Table 2. The correlation between E and the substituent constant (S) is shown in Fig. 3. The figure shows, in spite of the scatter of points, that the increase in the energy of activation tends to occur as the ester linkage becomes more reactive. This is the opposite to what might be expected and to the findings of Tommila & Hinshelwood (1938) whose results, plotted in the same way, are shown in Fig. 2.



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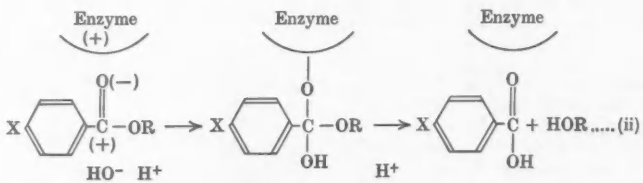

(IV)

The experimental values were not sufficiently accurate to determine values of A.

DISCUSSION

It is usually supposed that alterations in the structure of a substrate will affect the rate of an enzyme-catalysed reaction by altering the degree of contact between the enzyme and the substrate on which interaction depends; the possibility that structural alteration may change the intrinsic reactivity of the substrate is frequently overlooked. This work shows that the reactivity of a compound can be at least as important as the 'goodness of fit' between it and the enzyme.

It is at first sight anomalous that in a reaction involving the cleavage of a bond to give an overall reaction of type (i), E, the energy of activation, should tend to increase as the bond becomes more reactive. This anomaly may indicate that the temperature coefficient of the reaction is controlled by the formation of an intermediate compound between the enzyme and some activated part of the substrate molecule such as the carbonyl group.

The formation of such an intermediate compound is no new hypothesis. Wilson, Bergman & Nachmansohn (1950) have produced extensive evidence of the formation of compounds between esters and the cholinesterase from the electric organ of *Electrophorus*. The evidence given above favours the type of bond which would require greater energy of activation as the carbonyl group becomes activated by an electrophilic substituent in the benzene ring. This condition might best be fulfilled by the enzyme itself acting as an electrophilic reagent on the carbonyl oxygen which carries a partial negative charge giving a reaction mechanism according to scheme (ii).

Further light might have been thrown on this problem by a determination of Michaelis constants but, as has been shown above, this was not possible with the methods available.

SUMMARY

1. A series of derivatives of benzoylcholine has been prepared with substituents in the benzene ring, and the rate of hydrolysis of these compounds with cholinesterase of horse serum has been determined.

2. For this series it is concluded that the innate reactivity of the ester linkage is as important as the 'goodness of fit' between enzyme and substrate in determining the rate of reaction.

3. Solution of the Arrhenius equation suggests that greater energy of activation is required when the carbonyl group is the more activated. This condition could be fulfilled by supposing a positive or partially positive charge on the enzyme to react with the carbonyl oxygen of the ester to form an intermediate compound.

I should like to express my thanks to Dr W. F. Widdas, Dr T. S. Work and Sir Cyril Hinshelwood for their interest and helpful discussion during the course of this work, and to Mr D. Cragoe and Mr C. B. Hill, my assistants at The National Institute for Medical Research, London, where part of this work was performed.

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